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The Cystic Fibrosis Transmembrane Conductance Regulator in Essential Fatty Acid Metabolism

by

Farah Bhura-Bandali ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

in

Nutrition and Metabolism

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F Bhura-Bandali

11131 23A Avenue
Edmonton, Alberta
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis *entitled* ***The Cystic Fibrosis Transmembrane Conductance Regulator in Essential Fatty Acid Metabolism*** submitted by Farah Bhura-Bandali in partial fulfillment of the requirements for the degree of **Master of Science** in **Nutrition and Metabolism**.

Dr. MT Clandinin (Supervisor)

Dr. CJ Field

Dr. SFP Man

June 20, 1997

To my husband whose love, support and encouragement

made it all possible. Thanks Aly.

ABSTRACT

The majority of cystic fibrosis (CF) is caused by the ΔF508 mutation in the CF transmembrane conductance regulator (CFTR), leading to abnormal chloride transport. CF patients exhibit low essential fatty acid (C18:2n-6) levels in plasma and tissue lipids, independent of C18:2n-6 intake and pancreatic insufficiency. Fatty acid incorporation into phospholipids is influenced by chloride channels which suggests that the CFTR may regulate C18:2n-6 metabolism. The objective of this study was to determine if CFTRΔF508 reduces C18:2n-6 incorporation in phospholipids. Normal, CF and transfected CF cells were used to determine the lipid profile and ^{14}C18:2n-6 incorporation into cell lipids. CF cells exhibited low C18:2n-6 levels in phospholipids. ^{14}C18:2n-6 incorporation was reduced in phospholipids and increased in triacylglycerol of CF cells. Kinetic modelling of time course data for ^{14}C18:2n-6 incorporation revealed a loss of metabolic control over the intracellular partitioning of C18:2n-6 between phospholipid and triacylglycerol in CF. Normal gene expression increased ^{14}C18:2n-6 incorporation into phospholipids and triacylglycerol. These observations suggest that CF represents a defective utilization of C18:2n-6 which is explained in part by the CFTR.

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Table of Contents

			Page
List of Tables			
List of Figures			
Chapter I - Literature Review and Rationale			
A. Introduction			1
B. Biological Membranes			
	1.	Introduction	2
	2.	The Membrane Model	3
	3.	Membrane Lipids and Cell Function	5
	4.	Factors Affecting Fatty Acid Composition of Membrane Lipids	7
		a) Dietary Fat	8
		b) Fatty Acid Turnover	8
		c) Fatty Acid Desaturation-Elongation	10
		d) Chloride Channels	13
		e) Summary	14
C. Cystic Fibrosis			
	1.	Overview of the Disease	15
	2.	The Cystic Fibrosis Gene	16
	3.	The Major Mutation: ΔF508	16
	4.	Structure and Function of the CFTR	
		a) Structural Analysis of the CFTR	19
		b) Abnormal Electrolyte Transport	20
		c) CFTR: A cAMP-Regulated Chloride Channel	21
		d) Other Functions of the CFTR	21

5. Essential Fatty Acid Status in Cystic Fibrosis 22

a) Fat Malabsorption 23

b) Restriction of Dietary Fat 24

c) Increased Oxidation of Linoleic Acid due to Vitamin E Deficiency 25

d) Increased Utilization of Linoleic Acid for Energy 25

e) A Desaturation Defect in Linoleic Acid Metabolism 26

f) Impaired Arachidonic Acid Release 28

g) Summary 29

D. Rationale 30

E. Outline of Thesis

1. Hypothesis 31

2. Research Objectives 31

3. Thesis Organization 32

F. References 33

Chapter II - The ΔF508 Mutation in the CFTR Alters Control of C18:2n-6 Utilization in Cultured Epithelial Cells

A. Introduction 42

B. Analytical Methods and Statistical Analysis

1. Materials 44

2. Cell Lines and Maintenance 44

3. ^{14}C18:2n-6 Incorporation 45

4. Lipid Extraction 46

5. Thin Layer Chromatography 46

6. Formation of Fatty Acid Methyl Esters 47

7. Gas-Liquid Chromatography 47

8. Double Bond Separation 47

9. Liquid Scintillation Counting 48

10. ^{36}Chloride Efflux Assay 48

11. Statistical Analysis 49

C. Results

1. Fatty Acid Composition of PANC-1 and CFPAC-1 Cells 50

2. ^{14}C18:2n-6 Incorporation into Phospholipids and Triacylglycerol of PANC-1 and CFPAC-1 Cells 51

3. Time Course of the Incorporation of ^{14}C18:2n-6 into Triacylglycerol and Phospholipids of PANC-1 and CFPAC-1 Cells 52

4. The Effect of CFTR on ^{14}C18:2n-6 Incorporation into Phospholipids and Triacylglycerol 56

5. Conversion of ^{14}C18:2n-6 to its Desaturation and Elongation Products: Distribution of Labeled Fatty Acids into Phospholipids 61

D. Discussion 65

1. Transfected CFPAC-1 Cells in the Study of CFTR Function 65

2. Chloride Conductance of PANC-1 and CFPAC-1 Cells 66

3. Incorporation of Linoleic Acid in Phospholipid and Triacylglycerol 67

4. Total Linoleic Acid Incorporation into Cellular Lipids 68

5. Desaturation-Elongation of Linoleic Acid 69

6. Summary 72

E. References 73

Chapter III - General Summary and Discussion

A. Summary 77

B. Discussion 79

Appendix I- Essential Fatty Acid Composition of PANC-1 and CFPAC-1 Cells 81

List of Tables

		Page
Chapter II		
Table 1	The Conversion of C18:2n-6 to its Desaturation-Elongation Products in Phospholipid in PANC-1 and CFPAC-1 Cells	63
Table 2	Distribution of ^{14}C18:2n-6 Desaturation-Elongation Products in Phospholipids of PANC-1 and CFPAC-1 Cells	63
Table3	The Conversion of C18:2n-6 to its Desaturation-Elongation Products in Phospholipid of Transfected CFPAC-1 Cells	64
Table 4	Distribution of ^{14}C18:2n-6 Desaturation-Elongation Products in Phospholipids of Transfected CFPAC-1 Cells	64
Appendix I		
Table 1	Average C18:2n-6 and C20:4n-6 Composition of Phospholipids of PANC-1 and CFPAC-1 Cells	81

List of Figures

		Page
Chapter I		
Figure 1	The membrane model.	3
Figure 2	Metabolic turnover of phospholipid fatty acids.	9
Figure 3	Fatty acid desaturation and elongation.	12
Figure 4	The cystic fibrosis transmembrane conductance regulator (CFTR).	17
Figure 5	Defective maturation of the CFTR.	18
Chapter II		
Figure 1	C18:2n-6 and C20:4n-6 composition of CFPAC-1 cells in comparison to PANC-1.	50
Figure 2	^{14}C18:2n-6 incorporation into phospholipid and triacylglycerol of PANC-1 and CFPAC-1 cells.	51
Figure 3	^{14}C18:2n-6 incorporation into phospholipids of PANC-1 and CFPAC-1 cells.	52
Figure 4	Time course for incorporation of ^{14}C18:2n-6 over four hours into triacylglycerol and phospholipid of PANC-1 and CFPAC-1 cells.	53
Figure 5	Kinetic modeling of the rate constants for the incorporation of ^{14}C18:2n-6 over four hours into triacylglycerol and phospholipid of PANC-1 and CFPAC-1 cells.	54
Figure 6	Time course for incorporation of ^{14}C18:2n-6 over four hours into phospholipids of PANC-1 and CFPAC-1 cells.	55

		Page
Figure 7	Chloride conductance of transfected CFPAC-1 cells.	56
Figure 8	^{14}C18:2n-6 incorporation into phospholipid and triacylglycerol of transfected CFPAC-1 cells.	57
Figure 9	^{14}C18:2n-6 incorporation into triacylglycerol: a comparison between transfected, PANC-1 and CFPAC-1 cells.	58
Figure 10	^{14}C18:2n-6 incorporation into phospholipid: a comparison between transfected, PANC-1 and CFPAC-1 cells.	58
Figure 11	^{14}C18:2n-6 incorporation into phosphatidylcholine: a comparison between transfected, PANC-1 and CFPAC-1 cells.	59
Figure 12	Chloride conductance of PANC-1 and CFPAC-1 cells.	60
Figure 13	^{14}C18:2n-6 incorporation into phospholipids of transfected CFPAC-1 cells.	61

Chapter I

Literature Review and Rationale

A. Introduction

Biological membranes are highly selective permeability barriers responsible for numerous essential cellular functions (Yeagle, 1987). The proteins and lipids in the membrane function singularly and together to provide its structure and function. Hence, defects in these fundamental membrane components can have profound implications for the proper physiological functioning of the cell. Cystic fibrosis (CF) is an example of a fatal autosomal recessive disease in which a defective membrane protein in secretory epithelia causes excessive accumulation of mucus and other pathophysiological conditions (Smith, 1995). For decades, plasma and tissue lipids of patients with CF have been shown to exhibit essential fatty acid deficiency (Kuo et al., 1962; Lloyd-Still et al., 1981; Farrell et al., 1985; Parsons et al., 1988), the cause of which remains unclear. The transport protein, CF transmembrane conductance regulator (CFTR), is a cAMP-dependent chloride ion channel (Drumm et al., 1990; Bear et al., 1992) and chloride channels have been found to play a role in essential fatty acid metabolism (Kang et al., 1992). Whether the function of the CFTR includes regulation of essential fatty acid metabolism is not known. The knowledge of the functions of the CFTR is far from complete and considering the size, complexity and integral situation of this protein opens the possibility of its role, either direct or indirect, in essential fatty acid metabolism.

B. Biological Membranes

1. Introduction

Cell membranes participate in numerous critical functions of living cells (Yeagle, 1987). The past 25 years mark the development of a better understanding of the dynamics of biological membranes and their organization (Aloia et al., 1988). A range of analytical techniques have broadened the knowledge of the relationships between the structural and functional properties exhibited by a great diversity of membranes. Since the 1920s, several models including the "Lipoidal-Bilayer" concept of Gorter and Grendel in 1925 to the "Unit Membrane" of Robertson in the 1960s and "Fluid Mosaic" model of Singer and Nicolson in 1972, have attempted to correlate functional parameters with the architecture of membranes. Since these early models, considerable research has gone on to defining the multifaceted aspects of the structure and function of membranes. It has now become generally accepted that biological membranes are dynamic and responsive structures with regards to membrane constituents (Clandinin et al., 1985a; McMurchie, 1988; Spector and Yorek, 1985). Hence, in addition to characterizing the periphery of cells and subcellular organelles, biological membranes also contribute to their various intra- and extra-cellular functions.

2. The Membrane Model

The current model of biological membranes is based on the fluid mosaic model proposed by Singer and Nicolson (1972). This model has advanced the concept of membranes, allowing recognition of the membrane's dynamic nature (Clandinin et al., 1985a and 1991). It is now recognized that specific organizational heterogeneity serves functional, structural and possibly conductive roles. Lipids are arranged in a bilayer with two classes of proteins: peripheral proteins which are bound to the surface and integral proteins which are inserted to varying degrees in the membrane or span the membrane (Figure I.1). This structure results from the amphipathic nature of polar lipids characteristically found in biological membranes. Further, the lipid bilayer is anisotropic with well-ordered and liquid-crystal-like regions (Yeagle, 1989). The presence of integral membrane proteins has lead to the recognition of domains where lipid-protein and

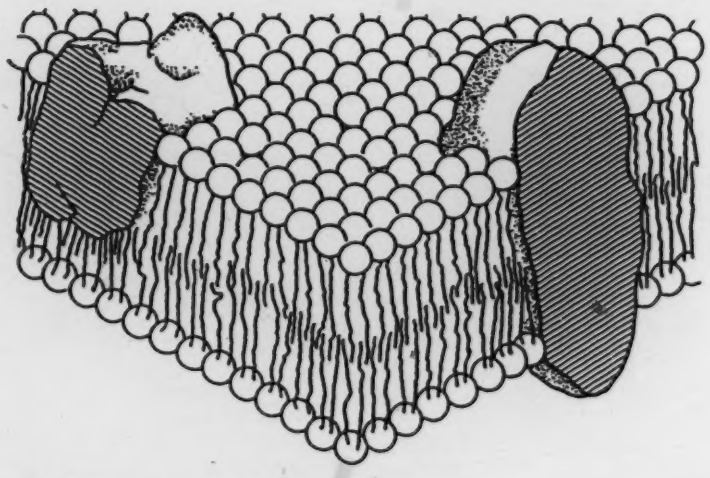

Figure I.1. The membrane model.
(Singer and Nicolson, 1972)

lipid-lipid interactions occur (Clandinin et al., 1985a and 1991; Curtain et al., 1988). These interactions are not only highly specific, but are highly organized to provide structural-functional characteristics of a particular membrane.

Although there are numerous varieties of lipids found in biological membranes, phospholipids are the principal form (Yeagle, 1987). Phospholipids are amphipathic polar lipids carrying charges which are held at the membrane surface. Classified based on the structure of their headgroup, phosphatidylcholine is among the most common of the phospholipids and is mainly situated in the outer layer. Other common membrane lipids include phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidlylglycerol. While phospholipids comprise the major class of lipids in cell membranes, glycosphingolipids are also present exclusively in the outer leaflet of the bilayer (Yeagle, 1987; Curtain et al., 1988). High levels of cholesterol are also found in cell membranes and has been shown to influence the structural and dynamic organization of the membrane.

Variety has been observed with respect to the hydrocarbon chains of phospholipids (Yeagle, 1987). Saturated and unsaturated fatty acids have been found to be esterified to the glycerol of phospholipids. The fatty acid composition is particular to the class of phospholipid and the membrane type. While saturated fatty acids are esterified to sn-1 position of the membrane phospholipid, unsaturated fatty acids are predominantly esterified to the sn-2 position. Furthermore, *cis* isomers of unsaturated fatty acids are the most common which perturbs the structure of the membrane more than saturated fatty acids. Hence,

alterations in the fatty acid composition of membrane phospholipids has the potential to alter membrane structure and properties.

Both lipids and proteins in the membrane have mobility (reviewed by Clandinin et al., 1985a and 1991; Yeagle, 1987). Lateral mobility is thought to be diffusion-limited, dependent on the fluidity of the bilayer and size of the protein. This lateral movement of integral proteins is considered a mechanism for regulation of specific cell surface properties. A second type of movement in membranes is transmembrane transport which applies to a variety of molecules including lipids. It consists of "flip-flop" which is slower compared to lateral movement and is thought to contribute to bilayer sidedness or asymmetry and membrane stability. A third type of movement is that of the fatty acyl chains of membrane phospholipids which is viewed as a potential mechanism for membrane fluidity.

3. Membrane Lipids and Cell Function

Membrane proteins are dependent on the lipid bilayer for their structure and function (Yeagle, 1987). The motion of enzyme proteins is thought to be a prerequisite for their function (Clandinin et al., 1985a) and are lipid-dependent (Stubbs and Smith, 1984) which suggests a close relationship between membrane proteins and lipids. While the lipid-lipid interactions determine physical properties of the membrane and depend on the composition of membrane lipids, the protein-lipid interactions allow the activity of the membrane to be modulated by changes in the physical properties of membrane lipids (reviewed by

McMurchie, 1988). The relationship of polyunsaturated fatty acids and membrane fluidity and function has been thoroughly reviewed (Stubbs and Smith, 1984; Brenner, 1984; Clandinin et al., 1985a and 1991; Spector and Yorek, 1985; Yeagle, 1989). Studies have shown that specific membrane composition with respect to lipid fluidity modulates the function of integral proteins (reviewed by Clandinin et al., 1985a; McMurchie, 1988). Specifically, this effect has been demonstrated with respect to ion channels (Van Hooqevest and DeKruijff, 1978), the insulin receptor (Field et al., 1990), thyroid hormone receptor (Venkatraman et al., 1986) and the adenosine receptor (Kang et al., 1992a).

Fatty acids have been shown to act both as modulators and messengers of signals triggered at the level of cell membranes (Sumida et al., 1993; Galli et al., 1993). This process is mediated by hydrolytic enzymes which include phospholipases (especially phospholipase A_2 and C) and act on membrane phospholipids. Phospholipase A_2 cleaves the acyl residue at the sn-2 position of glycerophospholipids, usually a polyunsaturated fatty acid, particularly arachidonic acid (C20:4n-6). Arachidonic acid metabolites, the eicosanoids, are produced through the combined action of phospholipase A_2 and the cyclooxygenase and lipoxygenase enzymes. These endogenously formed oxygen-carrying metabolites of arachidonic acid include prostaglandins, thromboxanes, leukotrienes and monohydroxyeicosatetraenoic acids (HETEs) (Chilton and Lichtenstein, 1990). The eicosanoids are physiologically active compounds involved in numerous bodily functions such as reproduction, immunity, skin integrity and pulmonary function (Yamanaka et al., 1981).

The action of phospholipase C cleaves the ester bond between glycerol and phosphoric acid, releasing diacylglycerol and phosphate-base residues (Sumida et al., 1993; Galli et al., 1993). The phospholipase C acting on phosphoinositides releases diacylglycerol and inositol triphosphate. While diacylglycerol stimulates protein kinase C, inositol triphosphate stimulates the release of calcium from intracellular stores. The activation of protein kinase C by diacylglycerol and unsaturated fatty acids has been suggested to cause physiological as well as pathophysiological effects (Merrill, 1992). While the lipid soluble products such as eicosanoids are involved in cell-cell interactions, most polar products including diacylglycerol and inositol triphosphate do not leave the cell, thus act intracellularly (Galli et al., 1993). Hence, changes in the fatty acid composition of membrane lipids and the subsequent release of lipid second messengers can have profound implications for cell regulation.

4. Factors Affecting Fatty Acid Composition of Membrane Lipids

A myriad of physiological processes are mediated by the types of unsaturated fatty acids esterified in membrane lipids (Sprecher, 1992). Regulation of the types of polyunsaturated fatty acids found in membrane lipids is due to the coordinated efforts of enzymes involved in the biosynthesis and the subsequent esterification of these fatty acids into specific phospholipids. Specifically, dietary fat, fatty acid turnover and fatty acid desaturation-elongation reactions play a role in the compositional alteration of fatty acids found in membrane lipids. Recent

evidence also suggests a role for membrane proteins such as chloride channels in the incorporation of fatty acids into membrane phospholipids (Kang et al., 1992c).

a) Dietary Fat

It is generally accepted that cell membrane lipid composition is influenced by the nature of dietary fat (reviewed by Clandinin et al., 1985a and 1991; Sprecher, 1992). Numerous animal studies utilizing a variety of organ systems have established that phospholipid components of cell membranes are dynamic in nature and can be compositionally altered by changes in the fatty acid makeup of a nutritionally adequate diet (Innis and Clandinin, 1981; Clandinin et al., 1985b; Garg et al., 1987 and 1990; Neelands and Clandinin, 1983; Field et al., 1990; Venkatraman et al., 1986; Kang et al., 1992a; Foot et al., 1982; Cinader et al., 1983). In vitro studies utilizing cells grown in culture have also shown that cells readily take up free fatty acids bound to albumin and/or other proteins from culture medium and incorporate them into membrane phospholipids and triacylglycerol (Rosenthal, 1987; Kang et al., 1992b and 1992c).

b) Fatty Acid Turnover

Membrane lipids are not static; they are in a dynamic state of turnover (Spector et al., 1981 and 1992). The turnover of fatty acids found in cell membrane lipids occurs via a remodeling pathway specifying deacylation-reacylation reactions (Sprecher, 1992). Activation of fatty acids by fatty acyl-CoA synthetase produces fatty acyl-CoA esters which can be incorporated into

phospholipids and triacylglycerols by the action of acyltransferase (acylation) (Thompson, 1992a). Fatty acyl-CoA synthetase has been shown to be a major regulatory step in the production of fatty acyl-CoA derivatives for incorporation into membrane phospholipids (Jeffcoat and James, 1984). Deacylation by phospholipase A releases the fatty acid from either sn-1 or sn-2 position through the action of phospholipase A_1 or A_2, respectively, leaving these sites available for re-esterification by another fatty acid (Holub and Kuksis, 1978; Sprecher, 1992). Hence, a constant metabolic turnover of fatty acids is maintained via the three enzymatic steps: 1) deacylation by phospholipase A_2; 2) fatty acid activation by fatty acyl-CoA synthetase; and 3) reacylation by acyltransferase (Figure I.2). This pathway elucidates how membrane phospholipids selectively acquire unsaturated fatty acids which are predominantly found in the sn-2 position of membrane phospholipids (Sprecher, 1992). Fatty acid incorporation can also

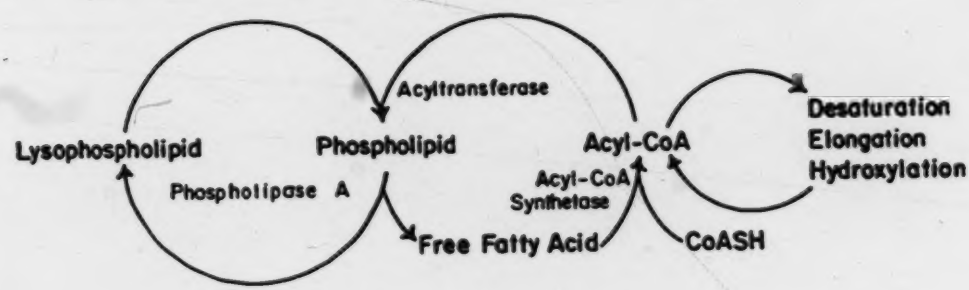

Figure I.2. Metabolic turnover of phospholipid fatty acids.
(Modified from Thompson, 1992b)

occur during the de novo synthesis of phospholipids (Clandinin et al., 1985a; Sprecher, 1992). However, the relative contribution of these two routes varies greatly with cell types and between individual fatty acids (Rosenthal, 1987).

Phospholipases are membrane-associated enzymes (van den Bosch, 1980) and it has been shown that both phospholipase A_1 and A_2 discriminate against molecular species of diacylglycerol phospholipids which have highly unsaturated fatty acids at the sn-1 and sn-2 positions, respectively (reviewed by Holub and Kuksis, 1978). Solubilized mitochondria phospholipase A_2 released oleic acid preferentially to arachidonic acid from rat liver phosphatidylethanolamine (Waite and Sisson, 1971). Similar specificity has been noted for phospholipase A_1 (reviewed by Holub and Kuksis, 1978). Fatty acyl chain length, degree of saturation and unsaturation and the nature of the phospholipid head group determine the specificity of acylating enzymes.

c) Fatty Acid Desaturation-Elongation

Fatty acyl chain desaturation and elongation are necessary steps for the synthesis of polyunsaturated membrane lipids (Jeffcoat and James, 1984). Prior to being incorporated into structural lipids, free fatty acids and those derived from diet are converted to fatty acyl CoA derivatives by fatty acyl-CoA synthetase. The resulting fatty acyl-CoA derivative is then available for chain desaturation and elongation as indicated in Figure I.2 above. Desaturation is associated with the endoplasmic reticulum where double bonds can be introduced at the $\Delta 4$, $\Delta 5$, $\Delta 6$ and $\Delta 9$ positions in most animal systems, but never beyond the $\Delta 9$ position

(reviewed by McMurchie, 1988 and Rosenthal, 1987; Mayes, 1993). The first double bond introduced into a saturated fatty acid is nearly always in the Δ9 position. Hence, the Δ9 desaturase enzyme system will catalyze the conversion of palmitoyl-CoA or stearoyl-CoA to palmitoleyl-CoA or oleyl-CoA, respectively (Figure I.3).

The fact that mammalian cells lack desaturase enzymes of the Δ12 or higher varieties implies that linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3) cannot be synthesized and must be obtained from diet (Jeffcoat and James, 1984; Sprecher, 1992; Simplicini and Valle, 1994). It is generally accepted that metabolism of these essential fatty acids, C18:2n-6 and C18:3n-3, lead to C22:5n-6 and C22:6n-3 as the final products by an alternating series of position-specific desaturases and malonyl-CoA dependent chain elongation steps. Specifically, the CoA derivatives of C18:2n-6 and C18:3n-3 are acted upon by a Δ6 desaturase to add a third and fourth cis double bond, respectively. This step has been shown to be a regulatory point for the final production of C20:4n-6. Although Δ4 desaturase has been indicated as a requirement for the last step, its presence has never been established (Voss et al., 1991). Further, the authors have shown that formation of 22:5n-6 and 22:6n-3 is in fact independent of Δ4 desaturase enzyme system and that an alternative retroconversion pathway is used as indicated in Figure I.3.

The Δ6 desaturase step has been shown to be a regulatory point for the production of arachidonic, docosapentaenoic and docosahexaenoic acids

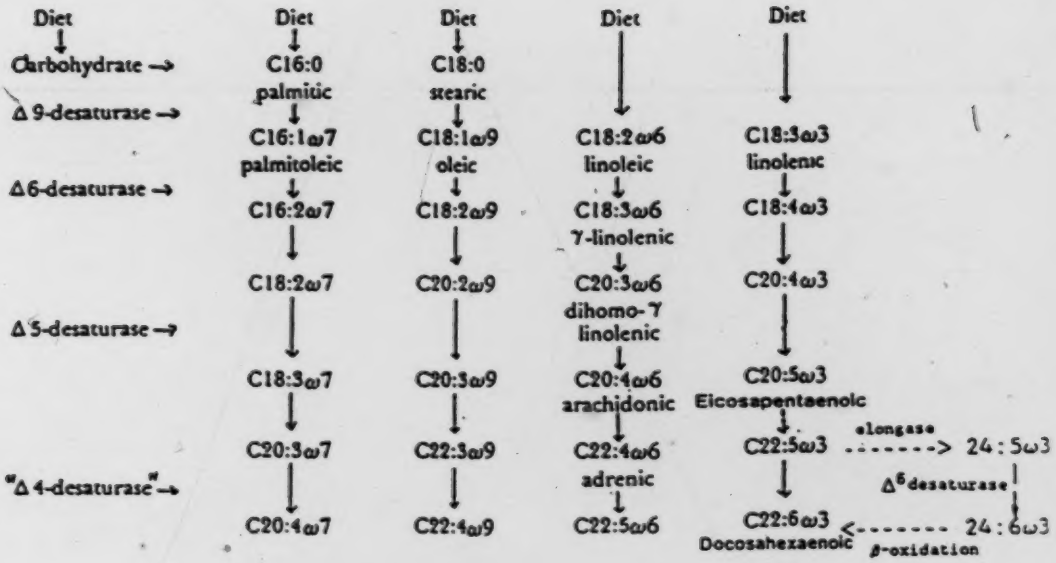

Figure I.3. Fatty acid desaturation and elongation.
(Modified from Kang et al., 1994)

(reviewed by McMurchie, 1988). Hence, many cultured cell types which exhibit endogenous synthesis of arachidonic acid retain active Δ6 desaturase activity (Rosenthal, 1987). Differences in the extent of endogenous C20:4n-6 synthesis are thought to mostly reflect differences in the extent of this enzyme activity. There is evidence to suggest that the lipid fluidity of endoplasmic reticulum membrane may itself direct the activity of Δ6 desaturase (reviewed by McMurchie, 1988). The activity of Δ6 desaturase has been shown to be influenced by several factors. It is decreased with aging, fasting, diabetes mellitus, glucose, ethanol, protein deficiency, saturated and trans fatty acids and

several hormones including epinephrine, thyroxine and glucocorticoids. ω3 fatty acids have also been shown to reduce Δ6 desaturase activity (Garg et al., 1988). It has been suggested that preference for desaturation by this enzyme is n-3 > n-6 > n-9.

The microsomal chain elongation adds two-carbon units to long chain fatty acids using the CoA derivatives as substrates (Bernert and Sprecher, 1979; reviewed in Rosenthal, 1987). The system has not been fully characterized in vivo, however it is thought that there are at least two different condensing enzymes; one for saturated substrates and another for unsaturated ones (Prasad et al., 1986). While the condensation step is considered rate-limiting for saturated as well as 18-C unsaturated primers, it remains unknown whether a single or distinct enzymes are involved in the chain elongation of 18-C vs 20-C polyunsaturated fatty acids. Deficiencies have not been identified for this process in cell culture systems. Further, both normal and transformed cells have been found capable of elongating palmitic acid (C16:0) to stearic acid (C18:0) and C18 polyunsaturated fatty acids to C20 fatty acids. It has been suggested that the process of elongation is rapid (Sprecher, 1992). In cells supplemented with ^{14}C18:2n-6 and ^{14}C18:3n-3, the intermediates ^{14}C18:3n-6 and ^{14}C18:4n-3 are barely detected (reviewed in Rosenthal, 1987). Because of its rapid nature, it is thought that elongation does not play a major role in regulating the synthesis of polyunsaturated fatty acids (Sprecher, 1992).

d) Chloride Channels

Can membrane proteins influence the fatty acid composition of membrane lipids? Much research has gone onto defining how lipids in membranes modulate the function of certain membrane proteins. Equally as important would be the observation of integral membrane proteins influencing the fatty acid composition of cell membrane lipids. Several different types of chloride channels exist in the plasma membrane as well as in intracellular organelles (Pusch and Jentsch, 1994). Chloride channels play critical functions in various aspects of cell physiology including volume regulation, transepithelial ion transport, stabilization of membrane potential, secretion and regulation of intracellular pH (Pusch and Jentsch, 1994; Valverde et al., 1995). Chloride channels are necessary for salt and water transport across epithelial cells. Kang and coworkers (1992c) have demonstrated that blocking chloride channels in normal human airway epithelial cells alters the fatty acid profile of membrane lipids. Specifically, impaired chloride conductance was found to reduce the incorporation of C18:2n-6 into phospholipids. This observation is not only unique, but expands the current knowledge of factors which have the potential to alter the fatty acid composition of membrane phospholipids. The exact mechanism(s) for this role of chloride channels remains unclear, however.

e) Summary

The issue of cell membrane lipid composition is complex. Competition between exogenous as well as intracellular fatty acid pools, cooperation of a

variety of enzyme systems, as well as proper functioning of membrane proteins such as chloride channels are important factors in the compositional alteration of membrane lipids. Further, the type of fatty acids found also play an important role in the proper functioning of the cell. Mammalian cells have a requirement for essential fatty acids (linoleic acid) and their metabolic products (arachidonic acid) which are both needed as structural membrane lipids and production of the chemically reactive eicosanoids. Thus, deficiencies in the any of the factors (dietary or biochemical) can have profound implications for the structure and function of biological membranes and subsequently the proper physiological milieu of the cell.

C. Cystic Fibrosis

1. Overview of the Disease

Cystic Fibrosis (CF) is the most common, often fatal, autosomal recessive disorder with a high incidence in Caucasian population (Jackson, 1989). It has a carrier rate of 1 in 20 and an incidence of approximately 1 in 2500 live births. At the cellular level, the disease is characterized by abnormalities in water and electrolyte transport. Clinically, CF leads to chronic pulmonary disease, pancreatic insufficiency and abnormal levels of electrolytes in the sweat (Tizzano and Buchwald, 1992; Quinton, 1990). Malnutrition and fulminant pulmonary infections were the cause of death in infancy for the majority of patients as described in the early reports of CF in the late 30s (Davis, 1993). The severity of the lung involvement still results in a high rate of morbidity and is often the cause

of death at an early age. However, remarkable progress has been made in the treatment of affected individuals during the past two decades allowing the median age of survival to rise from less than ten years to more than thirty years.

2. The Cystic Fibrosis Gene

CF was introduced into the medical literature as a disease entity over 40 years ago (Riordan et al., 1989). However, the gene for the defect was cloned and described in 1989 (Rommens et al., 1989; Riordan et al., 1989; Kerem et al., 1989) and encodes a polypeptide of 1480 amino acids and has a molecular mass of 168,138 daltons. Located on the long arm of chromosome 7 region q31, the gene expresses an integral membrane protein named the CF transmembrane conductance regulator (CFTR) (Figure I.4). The CFTR was termed the gene responsible for the disease for several reasons: 1) the gene correctly mapped the chromosomal region; 2) the gene was appropriately expressed in most epithelia affected by CF; 3) a mutation present on about 70% of the diseased chromosomes was found to be absent on non-CF chromosomes.

3. The Major Mutation: ΔF508

CF mutations are subtle changes at the nucleotide level. More than 500 different mutations have been described in the CFTR and while 20 commonly occur among Caucasians, others are rare with many being patient-specific (Tsui and Buchwald, 1991; Gray et al., 1995; Welsh et al., 1995). The most common

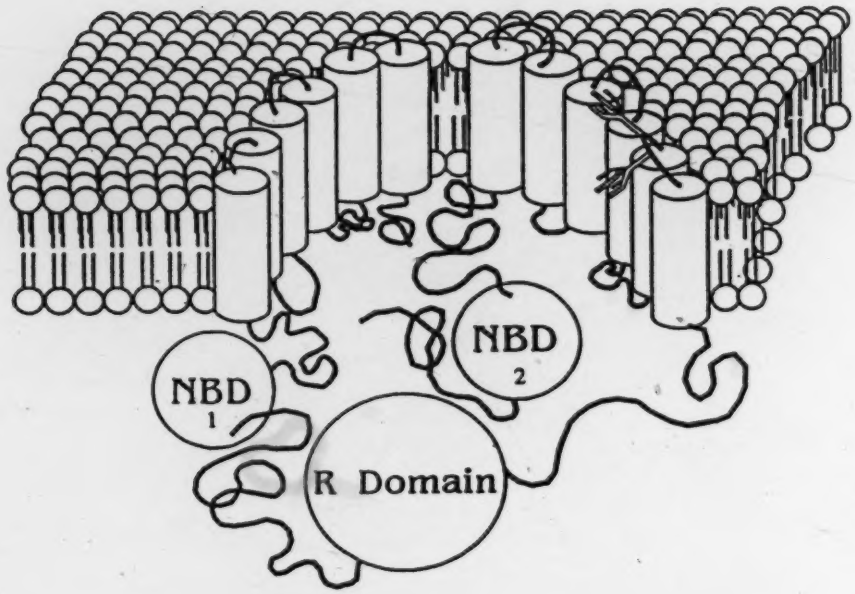

Figure I.4. The cystic fibrosis transmembrane conductance regulator (CFTR). Functional domains are indicated as follows: nucleotide (ATP) binding domain (NDB), regulatory domain (R Domain) and transmembrane domain (cylindrical regions). (Modified from Riordan et al., 1989)

mutation results in the deletion of a phenylalanine residue at position 508 (ΔF508) in the CF polypeptide and is located in NBD1 (see below) (Kerem et al., 1989; Riordan et al., 1989). ΔF508 accounts for 70% of all CF mutations and, in patients homozygous for this mutation, has been found to be clearly associated with the classic, severe CF with nearly universal pancreatic insufficiency as well as a high risk of meconium ileus (Kerem et al., 1989 and 1990a).

Several mutations including ΔF508 leads to mislocalization of the CFTR (Denning et al., 1992a; Kartner et al., 1992). Hence, the CFTRΔF508 is either

absent or present in decreased amounts in the apical membrane (Welsh and Smith, 1993). The CFTRΔF508 that does reach the plasma membrane, however, has been shown to form chloride channels with properties analogous to those of wild-type CFTR including the cAMP-dependent phosphorylation (Sheppard and Ostegaard, 1996). It has been suggested that this mislocalization occurs as a result of incomplete glycosylation (Figure I.5) (Cheng et al., 1990). Studies have demonstrated that partial glycosylation of the CFTR occurs as a result of modification of the nascent protein chain in the endoplasmic reticulum (Cheng et al., 1993). Additionally, the protein is not able to traffic further for complete glycosylation in the Golgi because it is unable to fold correctly (Cheng et al., 1990 and 1993; Thomas et al., 1992). Hence, impaired intracellular processing and transport of the CFTR appears to be the molecular basis for the majority of CF cases.

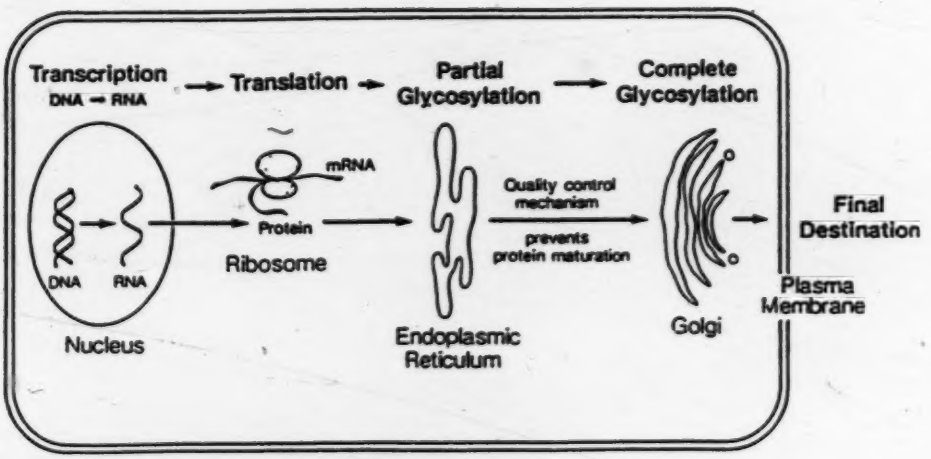

Figure I.5. Defective maturation of the CFTR.
(Modified from Cheng et al., 1993)

3. Structure and Function of the CFTR

a) Structural Analysis of the CFTR

The CFTR is composed of five domains: two membrane spanning domains (MSD), a regulatory domain (R) and two nucleotide binding domains (NBD) (reviewed by Riordan et al., 1989; Gray et al., 1995). Each of the MSDs consist of six hydrophobic transmembrane segments which form the ion channel pore. Situated in the cytosol, the R and NBDs possess regulatory functions. It has been suggested that, acting as a plug, the R domain closes the channel formed by the two MSDs. Opening of the channel requires phosphorylation of the R domain catalyzed by cAMP-dependent protein kinase A or protein kinase C (reviewed by Riordan et al., 1989; Tizzano and Buchwald, 1992; Gray et al., 1995). Although multiple consensus sites exist in the R domain for phosphorylation by protein kinase A, four are thought to be critical for complete channel activity (Welsh et al., 1994). Phosphorylation increases the net negative charge on the R domain believed to cause electrostatic repulsion of the domain from the internal face of the plasma membrane. In the absence of protein kinase A phosphorylation, increasing the net negative charge on the R domain by replacement of serine residues with aspartate or removal of the majority of the R domain leads to constitutively open channels. Activation of the CFTR also requires binding as well as hydrolysis of ATP which occurs at the two NBDs. It has been recently found that ATP binding and hydrolysis at NBD1 opens or activates the channel whereas at NBD2 closes or deactivates the channel (Carson et al., 1995). Thus, the NBDs possess distinct regulatory and functional roles in channel gating.

The unique structure of the CFTR has homology to the ATP-binding cassette superfamily of transporters (Widdicombe and Wine, 1991; Higgins, 1992; Welsh et al., 1993). Specifically, there are two main features which define this family of transporters: 1) putative NBDs which are the principal site of similarity among family members; 2) putative MSDs, especially the presence of six hydrophobic segments which span the membrane.

b) Abnormal Electrolyte Transport

The epithelia targeted by CF are relatively impermeable to chloride ion affecting the processes of absorption and secretion (Quinton, 1990; Welsh et al., 1993). Specifically, this abnormality disturbs the transport of salt and water across CF epithelia, modifying the quantity and composition of epithelial fluids. The evidence for defective chloride conductance observed in CF came from electrophysiological studies of the sweat gland and airway epithelia (Hanrahan et al., 1993). The chloride impermeability has been localized to the apical membrane of airway epithelia as might be expected for a chloride secreting epithelium (Willumsen and Boucher, 1989). A rise in cellular cAMP levels was shown to increase the apical chloride conductance of normal airway epithelia, but not CF airway. By contrast, chloride conductance is reduced in both the apical and basolateral membranes of the sweat gland which is consistent with chloride's role as a transepithelial shunt (Reddy and Quinton, 1989). The defective cAMP-dependent regulation of epithelial chloride channels has been found to be the all-

or-none difference between normal and CF cells in several organs (Welsh et al., 1993).

c) CFTR: A cAMP-Regulated Chloride Channel

Studies have confirmed that CFTR is a cAMP-regulated chloride channel. Expression of wild-type CFTR in CF epithelial cells was found to correct the chloride conductance abnormality (Drumm et al., 1990; Rich et al., 1990). Studies that followed examined the expression of the CFTR in non-epithelial cells which do not normally express this membrane protein and do not possess cAMP-activated chloride channels. Using the patch-clamp technique to assess chloride currents, cAMP agonists were found to increase chloride permeability in the cells expressing wild-type CFTR but did not alter current in non-transfected cells or those expressing CFTRΔF508 (Anderson et al., 1991; Kartner et al., 1991). Further, Bear and coworkers (1992) demonstrated that a regulated chloride channel with properties similar to that observed in intact cells was detected in planar lipid bilayers into which highly purified CFTR was incorporated.

d) Other Functions of the CFTR

While it is generally accepted that the CFTR is a cAMP-regulated chloride channel, this integral membrane protein may have other functions. It has been suggested that the CFTR may be involved in the direct regulation of other chloride channels (Welsh et al., 1993). A rise in cAMP levels may prompt the CFTR to insert chloride channels into the apical membrane, through mechanisms not yet

understood (Frizzell and Cliff, 1991). The CFTR may also indirectly regulate chloride channels by acting as a pump (Hyde et al., 1990). It has been speculated that the CFTR may pump some regulatory factor into or out of the cell thereby placing indirect controls on chloride channels. The possibility also exists that the CFTR may be associated with other membrane and/or cytoplasmic proteins (Welsh et al., 1993). Although controversial (Dunn et al., 1994), the function of intracellular acidification has also been proposed for the CFTR (Barasch et al.,1991; reviewed by Al Awqati, 1995). cAMP-mediated endocytosis and exocytosis were found to be absent in CF cells (Bradbury et al., 1992) indicating the involvement of CFTR in these important cellular functions. These authors and others (reviewed by Pusch and Jentsch, 1994 and Al Awqati, 1995) suggest that the CFTR may function as a chloride channel in intracellular organelles thereby by controlling the trafficking of the membrane and membrane-associated proteins. Clearly, the knowledge of the function of the CFTR is far from complete. Given the fast pace of research in this area, it is reasonable to expect exciting new discoveries regarding the functions of this unique membrane protein.

4. Essential Fatty Acid Status in Cystic Fibrosis

Abnormal essential fatty acid status in CF has been documented since the early sixties (Kuo et al., 1962). While the area of linoleic acid deficiency in CF is controversial (Biggeman et al., 1988), much of the literature supports its existence (Kuo et al., 1962; Gibson et al., 1986; Lloyd-Still et al., 1981; Farrell et al., 1985; Chase and Dupont, 1978; Dodge, 1975; Rosenlund, 1977; Hubbard and Dunn,

1980; Hubbard, 1983; Rogiers, 1983 and 1984; Parsons et al., 1988). The mechanism(s) for a linoleic acid deficiency in CF have not been clearly established. Several factors have been suggested to contribute to the low linoleic acid status observed in CF:

a) Fat Malabsorption

Eighty-five to ninety percent of the CF patients malabsorb fat due to pancreatic insufficiency (Orenstein, 1989a and 1989b; Collins, 1992). Consequently, the normal digestive process is impaired and patients often present with steatorrhea (Mearns, 1993). To combat fat malabsorption, past dietary regimes encouraged restriction of dietary fat. However, with the use of newer pancreatic enzyme supplements in the treatment of CF (Cannella et al., 1993), dietary fat is no longer restricted. Nonetheless, despite improvements in the potency and dose schedule of enzyme supplements, many patients still exhibit some steatorrhea (Pencharz and Durie, 1993).

Fat malabsorption can lead to abnormally low plasma levels of essential fatty acid (Gourley et al., 1982). However, this relationship does not appear to be a major factor precipitating linoleic acid deficiency in CF. CF patients receiving sufficient pancreatic enzyme supplementation to correct fat malabsorption still exhibited low plasma linoleic acid concentrations (Lloyd-Still et al., 1981; Gibson et al., 1986). Low linoleic acid levels in plasma phospholipids were also demonstrated in a sub-population of pancreatic sufficient CF patients (Hubbard et al., 1977; Rogiers et al., 1983; Parsons et al., 1988). Although pancreatic

insufficiency and the resulting fat malabsorption could contribute to a decreased availability of linoleic acid, this does not appear to be the main cause in CF.

b) Restriction of Dietary Fat

Anorexia, resulting in insufficient intake of energy and essential fatty acids often accompany the frequent infections experienced by patients with CF (Ramsey et al., 1992). However, dietary supplementation with linoleic acid has not consistently resulted in an improved status. Supplementation with safflower oil (approximately 80% linoleic acid) at a dose of 1g/kg/day did not increase the linoleic acid content of plasma phospholipids in CF patients (Lloyd-Still et al., 1981). In this study, adequacy of the overall energy intake of CF patients was not addressed. Insufficient energy intake to support adequate growth and maintain body weight can lead to abnormalities in essential fatty acid status. Parsons and coworkers (1988) have demonstrated abnormally low levels of linoleic acid in plasma lipids of CF subjects despite intake above the recommended level (3% of total energy). Additionally, although nutrition rehabilitation improved linoleic acid levels of CF subjects, they failed to reach normal ranges. It has recently been reported that in CF patients exhibiting ideal body weights, linoleic acid levels in plasma lipoprotein lipids were significantly lower than the non-CF group (Clandinin et al., 1995). It is possible that suboptimal energy intake may exacerbate linoleic acid deficiency in CF. A possibility also exists that CF patients may have requirements much greater than the recommended levels.

Nevertheless, the dietary factor alone does not seem to explain the low linoleic acid status of CF.

c) Increased Oxidation of Linoleic Acid Due to Vitamin E Deficiency

Vitamin E is an important antioxidant and a reduction in vitamin E status has been shown to cause an increase in the oxidation of polyunsaturated fatty acids (Machlin, 1991), including linoleic acid (Clement and Bourre, 1993). A deficiency of vitamin E often accompanies chronic fat malabsorption syndromes (Gourley et al., 1982), however vitamin E deficiency has not been consistently demonstrated in CF patients with fat malabsorption (Biggemann et al., 1988; Farrell et al., 1985). Further, in CF patients with malabsorption and vitamin E deficiency supplemented with 100-200 IU of α-tocopheryl acetate, there was no difference found with respect to the extent of linoleic acid deficiency (Farrell et al., 1985). Thus, while a linoleic deficiency may be exacerbated by a deficiency of vitamin E, this factor does not seem likely as the primary cause of the low linoleic acid level found in CF.

d) Increased Utilization of Linoleic Acid for Energy

In conditions of illness, there is a shift in metabolism to a greater use of lipid as energy substrate (Nanni et al., 1984). It has also been shown that there is an increase in basal metabolic rate (BMR) and protein turnover during periods of infection (Morton et al., 1988). This suggests that during periods of increased metabolic demands, polyunsaturated fatty acids (linoleic acid) may be used as an

alternate energy source. CF is a chronic disease characterized by recurring respiratory infections and periods of anorexia (Ramsey et al., 1992 Mearns, 1993). CF patients in energy deficit exhibit increased energy expenditure which has been found to be correlated with advanced lung disease (Pencharz et al., 1984). Above normal resting energy expenditure (REE) was reported in CF subjects and was found to inversely correlate with pulmonary function and nutritional status (% body fat) (Vaisman et al., 1991). An increase in REE was not observed in healthy CF patients who were adequately nourished (weight as % of ideal weight for height) with good pulmonary function (Fried et al., 1991). It is possible that increased energy demands enhance the utilization of fatty acids during periods of insufficient energy intake and/or pulmonary infections. Research using animal models suggests that there may be preferential oxidation of non-essential fatty acids when the body must make greater use of lipid fuels (reviewed by Field and Clandinin, 1984). Preferential oxidation of oleic and linoleic acids have been shown in studies using human subjects in energy balance (Jones et al., 1985). This suggests that during periods of metabolic stress, preferential use of non-essential fatty acid could occur, thereby sparing the essential fatty acid, linoleic acid. Although increased energy needs could exacerbate a linoleic acid deficiency, this is unlikely the primary cause in CF.

e) A Desaturation Defect in Linoleic Acid Metabolism

Desaturase enzymes involved in the metabolism of linoleic acid are the Δ6- and Δ5-desaturase (Sardesai, 1992; Simplicini and Valle, 1994) (Figure I.3).

Arachidonic acid can be sythesized from linoleic acid and is considered an essential fatty acid whereas linoleic acid can be differentiated as the essential dietary precursor. If the desaturation pathway functions normally, it would be predicted that a deficiency of C18:2n-6 would cause a decrease in the availability of C20:4n-6. Several studies have reported normal C20:4n-6 despite low levels of C18:2n-6 in CF (Chase and Dupont, 1978; Hubbard et al., 1977; Lloyd-Still et a., 1981; Hubbard and Dunn, 1980; Rogiers et a., 1984; Farrell et al., 1985; Biggeman et al., 1988; Lawrence and Sorrell, 1993) which argues against any metabolic defect in the desaturase enzymes. However, since C18:2n-6 is an obligatory requirement for the production of C20:4n-6 and is reduced in CF despite normal to even high (Christophe et al., 1992) C20:4n-6 levels, it may be argued that some factor may be increasing Δ6-desaturase activity which would lead to low levels of C18:2n-6 with normal to high levels of C20:4n-6. Speculations with respect to the role of desaturases are largely based on the assessment of the relative fatty acid profiles of plasma lipids and tissues in CF. Recently, quantitative measures of plasma lipoprotein fatty acid pools reported low levels of both linoleic and arachidonic acids in CF subjects (Clandinin et al., 1995), although normal quantitative pools of arachidonic acid have also been found in linoleic acid deficient plasma lipid fraction (Parsons et al., 1988). Studies directly examining the desaturation-elongation of C18:2n-6 in CF are lacking, indicating the need for more research to clarify the possible role of the desaturase enzymes in producing the characteristic essential fatty acid profile of CF.

f) Impaired Arachidonic Acid Release

It has been suggested that essential fatty acid deficiency instigates the primary symptoms in CF and that this results from an increased release of arachidonic acid and/or its metabolites (Strandvik, 1992). Arachidonic acid and its metabolites, prostaglandins, thromboxanes and leukotrienes, have been shown to regulate chloride transport in both healthy and CF epithelial cells (Eling et al., 1986; Leikauf et al., 1986; Anderson and Welsh, 1990; Calderaro et al., 1991). Also, prostaglandins, leukotrienes and hydroxyeicosatetraenoic acids (HETE) increase production of mucus in both the gastrointestinal and respiratory tracts (Marom et al., 1981; Lamorte et al., 1986). Regulation of the β-adrenergic response has also been shown to be influenced by arachidonic acid (Iizuka et al., 1986) and failure of β-adrenergic stimulation to increase ATP in CF has been reported (Davis, 1986). In CF, the release of arachidonic acid occurs at a steady rate, irrespective of the factors normally known to inhibit its release such as dexamethasone (Carlstedt-Duke et al., 1986). Also, there is evidence to suggest an increased concentration of arachidonic acid metabolites in CF (Chase and Dupont, 1978; Stead et al., 1986). It has been hypothesized that this increase in arachidonic acid release and the subsequent production of its metabolites results from defective regulation of phospholipase A_2, an enzyme which is also rate-limiting in the synthesis of eicosanoids (Strandvik, 1992).

Essential fatty acid deficiency is characterized by low plasma levels of linoleic acid and its metabolite arachidonic acid (Lefkowith et al., 1985; Brenner et al., 1981; Menon and Dhopeshwarkar., 1982) and a "true" essential fatty acid

deficiency would be expected to lead to lower levels of arachidonic acid and a decreased production of eicosanoids (Mathias and Dupont, 1985). Although low plasma and tissue lipid levels of linoleic acid are common to CF, their levels of arachidonic acid are normal (Chase and Dupont, 1978; Lloyd-Still et al., 1981; Hubbard and Dunn, 1980; Rogiers et al., 1984; Farrell et al., 1985; Parsons et al., 1988; Biggeman et al., 1988; Lawrence and Sorrell, 1993). Also, the synthesis of eicosanoids is greater than in control subjects (Chase and Dupont, 1978; Stead et al., 1986). While an impaired regulation of phospholipase A_2 can result in increased release of arachidonic acid and the subsequent production of eicosanoids, it remains unclear why the level of arachidonic acid in the plasma and membrane lipids of CF have been found to be normal and why this level remains so despite abnormally low levels of linoleic acid. It is indisputable, however, that despite linoleic acid deficiency normal levels of arachidonic acid, its increased release and the subsequent elevated production of eicosanoids suggest defective regulation of essential fatty acid metabolism.

g) Summary

While known for numerous decades, the incessant occurrence of essential fatty acid deficiency in CF was largely ignored because it had been considered a result of pancreatic insufficiency (Strandvik, 1992). It is clear that the nutritional consequences of CF alone cannot explain the typical low linoleic acid level found in the plasma and membrane lipids of CF patients. Further, the characteristic normal arachidonic acid level, its increased release and the subsequent elevated

production of eicosanoids suggests defect(s) in the control of essential fatty acid metabolism. Whether this is a primary defect as suggested by Strandvik (1988) or a secondary result of the defective membrane protein encoded by the CF gene remains unknown.

D. Rationale

Although essential fatty acid deficiency in CF has been documented since the 1960s (Kuo et al., 1962), its cause remains unclear. A review of the factors proposed to contribute to the low linoleic acid level indicates that the nutritional consequences of CF alone cannot explain the typical essential fatty acid profile. Furthermore, evidence suggests that the reduced linoleic acid level in CF is unlike the 'classic' essential fatty acid deficiency in which low linoleic acid levels are accompanied by low levels of arachidonic acid and its metabolites. Rather, it appears that in CF there is impaired regulation of essential fatty acid metabolism leading to normal levels of arachidonic acid despite decreased levels of linoleic acid. Active incorporation of fatty acids is a dynamic process which defines the types of fatty acids found in cell lipids and has been shown to be influenced by chloride channels. Specifically, blocking chloride channels was found to reduce the incorporation of fatty acids into membrane lipids (Kang et al., 1992) which implies that the faulty chloride channel in CF (the CFTR) may be involved in producing the distinct essential fatty acid profile of the disease.

The objective of the present research is to examine if the CFTRΔF508 reduces the incorporation of linoleic acid into membrane lipids of CF pancreatic epithelial cells.

E. Outline of Thesis

1. Hypothesis

It is hypothesized that the essential fatty acid (C18:2n-6) composition of CF phospholipids is influenced by the defective cystic fibrosis transmembrane conductance regulator (CFTRΔF508). More specifically, it is hypothesized that:

1. The C18:2n-6 content of phospholipids of CF will be lower in comparison to control cells.
2. The CFTRΔF508 will reduce the incorporation of C18:2n-6 into phospholipids of CF cells.
3. Transfection with wild-type CF gene will increase the incorporation of C18:2n-6 into phospholipids of CF cells.

2. Research Objectives

The first hypothesis posed will be examined by the extraction of lipid to determine the fatty acid composition of control and CF cells grown in standard culture conditions. Specifically, C18:2n-6 and its desaturation product, C20:4n-6, contents of total phospholipid, phosphatidylcholine, phosphatidylserine,

phosphatidylinositol and phosphatidylethanolamine will be analyzed from control and CF cells.

The second and third hypotheses will be investigated by utilizing ^{14}C18:2n-6 as the substrate for incorporation into cellular lipids of control, CF and CF cells transfected with the wild-type or CF (CFTRΔF508) genes. Lipids will be extracted and the radioactivity incorporated into triacylglycerol, total phospholipid, phosphatidylcholine, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine will be examined.

3. Thesis Organization

Chapter I comprises an in-depth review of biological membrane structure and function and CF in relation to essential fatty acid metabolism. Chapter II is presented in a paper format which includes the methods employed and results of the study examining the role of the cystic fibrosis transmembrane conductance regulator (CFTR) in essential fatty acid metabolism. Chapter III presents a general discussion and conclusions of the study with future research implications.

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Chapter II

The ΔF508 Mutation in the CFTR Alters Control of C18:2n-6 Utilization in Cultured Epithelial Cells

A. Introduction

Numerous essential cellular functions depend on biological membranes (Yeagle, 1989). Proteins and lipids exhibit both independence and synergy in the maintenance of membrane structure and function. Hence, defects in these fundamental membrane components can have profound implications for the proper physiological milieu of the cell. These observations have relevance to cystic fibrosis (CF), a fatal disease which exemplifies defects in both these important membrane components. CF is the most common autosomal recessive disease in Caucasians (Smith, 1995). At the cellular level, the disease is characterized by abnormalities in water and electrolyte transport. Clinically, CF leads to chronic pulmonary disease, pancreatic insufficiency and abnormal levels of electrolytes in the sweat (Tizzano and Buchwald, 1992; Quinton 1990; Smith, 1995). The gene responsible for CF encodes a membrane protein, CF transmembrane conductance regulator (CFTR) which is a cAMP-dependent chloride channel (Drumm et al., 1990; Anderson et al., 1991; Bear et al., 1992) with other regulatory functions (Hyde et al., 1990; Bradbury et al., 1992), thought to contribute to the pleiotropic nature of the disease. Genetic analysis has revealed over 500 mutations in the CFTR with the deletion of a phenylalanine residue at position 508 (ΔF508) being the most common, comprising nearly 70% of the diseased chromosomes (Rommens et al., 1989; Riordan et al., 1989; Kerem et al., 1989). A large majority of the patients homozygous for ΔF508 exhibit pancreatic insufficiency with heightened disease severity.

For decades, plasma and tissue lipids of patients with CF have been shown to exhibit low essential fatty acid (linoleic acid) levels (Kuo et al., 1962; Farrell et al., 1985; Lloyd-Still et al., 1981; Clandinin et al., 1995), the basis of which remains unclear. Reduced linoleic acid (C18:2n-6) level despite pancreatic sufficiency and adequate dietary supplementation have been reported (Lloyd-Still et al., 1981; Parsons et al., 1988; Clandinin et al., 1995) which argues against a nutritional cause. Despite low linoleic acid levels, normal to increased levels of arachidonic acid (C20:4n-6) occur in plasma and tissue lipids and the production of eicosanoids is elevated in CF (Lloyd-Still et al., 1981; Hubbard and Dunn, 1980; Carlstedt-Duke et a., 1986; Stead et al., 1986; Chase and Dupont, 1978; Christophe et al., 1992). Thus, it appears that the characteristic essential fatty acid profile of CF is unlike the 'classic' essential fatty acid deficiency but rather implies a defective regulation of essential fatty acid metabolism. Active incorporation of fatty acids is a dynamic process which defines the types of fatty acids found in cell lipids (Sprecher, 1992) and has been shown to be influenced by chloride channels (Kang et al., 1992). Inhibition of chloride channels was found to reduce essential fatty acid incorporation into phospholipids (Kang et al., 1992) which proposes a relationship between the function of the CFTR and essential fatty acid metabolism. Thus, the objective of the present study was to examine if the ΔF508 mutation in the CFTR reduces the incorporation of C18:2n-6 into membrane lipids of cultured CF pancreatic epithelial cells.

B. Analytical Methods and Statistical Analysis

1. Materials

Radioactive materials [1-^{14}C]C18:2n-6 (50mCi/mmol) and [$^{36}Cl^-$]NaCl in aqueous solution (12.7mCi/g chlorine) of more than 90% purity were purchased from NEN Research Products (DuPont Canada Inc., Mississuaga, ON) and used without further purification. Unlabelled fatty acid C18:2n-6 , lipid standards and other biochemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA) and Gibco BRL (Gaithersburg, MD, USA). All organic solvents were redistilled prior to use. Cystic fibrosis and control human pancreatic epithelial cell lines of ductal origin: CFPAC-1 (Schoumacher et al., 1990) and PANC-1 (Leiber et al., 1975) were purchased from ATCC (Rockville, MD, USA). CFPAC-1 cell lines transfected with wild-type CF gene or CF (ΔF508) gene (Drumm et al., 1990) were generously provided by Dr. RA Frizzell (Pittsburg, PA, USA).

2. Cell Lines and Maintenance

Cells lines were suspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% or 10% (v/v) fetal bovine serum (FBS) and Antibiotic-Antimycotic solution (Gibco BRL, Gaithersburg, MD, USA) containing 10,000 units penicillin G, 10,000*u*g streptomycin sulfate and 25*u*g/ml amphotericin B. Cells were plated on 25cm^2 tissue culture and 35mm tissue culture plates (chloride efflux) and cultured at 37°C in 5% CO_2 at 98% relative humidity in a tissue culture incubator (Model 3326, Forma Scientific). Culture

medium was changed every 2-3 days until confluent monolayers were formed which were utilized for biochemical assays.

3. ^{14}C18:2n-6 Incorporation

Confluent monolayers of cells were washed with Hank's Balanced Salt Solution (HBSS) and detached from the tissue culture flask surface with 0.25% (w/v) trypsin-EDTA (1mM EDTA.4Na) solution. The cell suspension was neutralized with culture medium containing FBS and collected in a centrifuge tube and spun at 1000rpm at 25°C for 3min. Cells were then washed once with medium without FBS and an aliquot removed for cell counts and viability. Using a hemocytometer, the average number of cells and viability per experiment was determined to be 2.0 - 3.0 x 10^6 and 85 - 90%, respectively. Cells were replated onto 60 or 100mm diameter tissue culture plates in 2ml medium without FBS. A mixture of labeled and unlabelled fatty acids suspended by sonication in sterile 5% (w/v) bovine serum albumin was added in a 100ul volume to give a final fatty acid concentration of 50uM and 1uCi of [1-^{14}C]C18:2n-6. Cells were incubated for various time points (0-4 hours) under conditions described above. Upon completion of incubation, cells were harvested using a rubber policeman and plates were washed twice with HBSS. Culture medium plus washes were removed by centrifugation and cell pellets were further washed twice with HBSS. Cell pellet suspended in 1ml HBSS was then used for lipid extraction and analyses.

4. Lipid Extraction

Cellular lipid was extracted with 20ml chloroform/methanol (2:1, v/v) (Folch et al., 1957) containing 0.005% (v/v) etoxiquin as the antioxidant. After vigorous mixing, 0.05% (w/v) calcium chloride was added, contents mixed and refrigerated overnight at 4°C. The chloroform phase containing lipid was collected, dried under a gentle stream of nitrogen and stored in sealed tubes at -70°C until further analysis.

5. Thin Layer Chromatography

Lipid classes were separated using thin layer chromatography (TLC). TLC plates were cleaned with hexane and activated at 110°C for at least 60 minutes. TLC tanks were lined with Whatman #1 filter paper and equilibrated with solvent systems prior to separation of neutral and phospholipids. Total phospholipid and triacylglycerol were separated using 250*u*m Silica Gel G plates (20 x 20cm, FisherScientific, Ottawa, ON) in a solvent system comprising petroleum ether:diethyl ether:acetic acid (160:40:2, v/v/v) for 25-30 minutes (Skipski and Barday, 1969). Individual phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine) were separated on 250*u*m Silica Gel H plates (20 x 20) in chloroform:methanol:2-propanol:triethylamine:0.25% (w/v) potassium chloride (60:18:50:36:12, by volume) solvent system for 90 minutes (Touchstone et al., 1980).

6. Formation of Fatty Acid Methyl Esters

Fatty acids were methylated using 14% (w/v) boron trifluoride/methanol reagent in hexane and allowed to reflux at 110°C for 60 minutes (Morrison and Smith, 1961). Neutral lipid (triacylglycerol) was saponified prior to methylation with 0.5N methanolic potassium hydroxide and allowed to reflux at 110°C for 60 minutes. When cooled to room temperature, water was added to methylated samples, contents were mixed and refrigerated overnight at 4°C. The upper hexane layer was extracted and concentrated in a Speed Vac Concentrator (Model 200H, Savant).

7. Gas-Liquid Chromatography

Fatty acid composition of phospholipids was carried out by a fully automated Varian Vista 6000 gas-liquid chromatograph equipped with a flame-ionization detector (Hargreaves and Clandinin, 1987). The analytical conditions used separated all saturated, mono- and poly-unsaturated fatty acids ranging from C14 to C24. Percentage area for all resolved peaks was analyzed using the Varian Vista 654 data system.

8. Double Bond Separation

Argentation TLC was used to resolve fatty acid methyl esters based on the degree of unsaturation (Suh et al., 1994). [1-^{14}C]C18:2n-6 labeled samples were applied on TLC plates impregnated with $AgNO_3$. 250μm Silica Gel H plates (20 x 20) were developed in a 10% (w/v) $AgNO_3$ solution in water in a TLC tank for 60

minutes. The plates were dried for 3 minutes in the dark and activated in a 110°C oven for 1 hour. Each sample and standards were spotted on the plate in a narrow band and the plates were developed twice in a solvent system of hexane:diethyl ether:acetic acid:toluene:acetone (50:4:2:40:4 by volume) for 1 hour and then for 30 minutes. Plates were dried at room temperature for 3 minutes and standards visualized with 0.1% (w/v) 2'7'-dichlorofluorescein in 95% (w/v) ethanol. Bands of fatty acids from saturated to those containing five double bonds were separated using this method.

9. Liquid Scintillation Counting

[1-^{14}C]$C_{18:2n-6}$ labeled samples separated by TLC were scraped directly into 20ml plastic scintillation vials and 10ml scintillation cocktail (ScintiSafe Econo 1, Fisher Scientific) was added. Radioactivity was counted in a Beckman LS 5801 Liquid Scintillation Counter with a counting efficiency of 94-95%. Quench was monitored by the "H-number" method.

10. Chloride Efflux

Efflux of radiolabeled chloride was used to assess the chloride conductance of the cell lines (Dunn et al., 1994). Cells grown to confluence on 35mm culture dishes were washed with bicarbonate free Krebs Ringer solution, loaded with 2.7μCi of [$^{36}Cl^-$]NaCl(aq) and incubated for 2hrs at 37°C in 5% CO_2 at 98% relative humidity in a tissue culture incubator (Model 3326, Forma Scientific). Following incubation, the monolayer was washed four times and

isotope efflux was measured by replacing 1ml Krebs Ringer solution at 15 second intervals. After 8 baseline measurements, 10*u*M forskolin and 100*u*M 3-isobutyl-1-methylxanthine (IBMX) was added and an additional 7 stimulated measurements were taken. Chloride efflux was calculated as $^{36}Cl^-$ released from the monolayer over time and presented as the Σ released (stimulated)/Σ released (baseline).

11. Statistical Analysis

Differences between PANC-1 (control) and CFPAC-1 (CF) cells or CFPAC-1 cells transfected with wild-type CF gene and CF gene was determined using Student's *t* test procedure with $p<0.05$ considered statistically significant. Data from time course experiments was also analyzed using the SAAM II Biological Modeling Program (Seattle, WA) which has been designed to illustrate the movement of tracer material between different biological compartments. The program utilizes simple differential equations that are solved and fitted using built-in mathematical and statistical functions (least squares fitting). SAAM II determined the rate constants for the movement of $^{14}C18:2n-6$ between lipid fractions (phospholipid and triacylglycerol) of PANC-1 and CFPAC-1 cells. Significant differences between the rate constants were also determined using Student's *t* test.

C. Results

1. Fatty acid Composition of PANC-1 and CFPAC-1 Cells[1]

The characteristic essential fatty acid profile of phospholipids from CFPAC-1 cells in comparison to PANC-1 cells indicates that these two cell types maintain quite different membrane C18:2n-6 levels (Figure II.1). C18:2n-6 was reduced in phosphatiylcholine and phosphatidylethanolamine while increased in phosphatidylserine of CFPAC-1 cells (77%, 39% and 186% of PANC-1, respectively). C20:4n-6 level in CFPAC-1 phosphatidylcholine, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine was found to be higher than PANC-1 (101%, 191%, 205% and 153%, respectively). These results are in agreement with those found for CF in previous

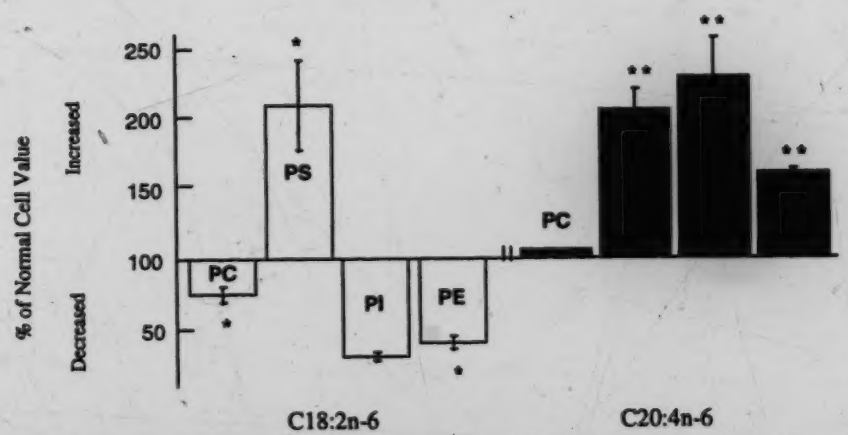

Figure II.1. C18:2n-6 and C20:4n-6 composition of CFPAC-1 cells in comparison to PANC-1. Lipid was extracted from confluent monolayers of cells and phospholipids separated by TLC. Fatty acids were methylated then subjected to gas chromatography. Values are the mean ± SEM, n=4. *p<0.05; **p<0.002.

[1] For clarity in the presentation of results, the cell types used in these studies are labeled PANC-1 (control), CFPAC-1 (cystic fibrosis), wild-type gene (CFPAC-1 cells transfected with wild-type CFTR) and CF gene (CFPAC-1 cells transfected with CFTRΔF508).

studies (Chase and Dupont, 1978; Farrell et al., 1985; Parsons et al., 1988; Levy et al., 1989; Christophe et al., 1992). Values for C18:2n-6 and C20:4n-6 composition of cells are also presented (Table 1, Appendix I).

2. ^{14}C18:2n-6 Incorporation into Phospholipids and Triacylglycerol of PANC-1 and CFPAC-1 Cells

Incorporation of essential fatty acid into lipids of CFPAC-1 cells exhibiting the ΔF508 mutation is distinct when compared to PANC-1 cells. The incorporation of labeled C18:2n-6 into total phospholipid was reduced (5.6% ± 1.61 vs 11.1% ± 1.42, $p<0.05$) while into triacylglyerol it was increased (5.5% ± 1.73 vs 1.2% ± 0.16, $p<0.05$) in CFPAC-1 cells (Figure II.2). Separation of

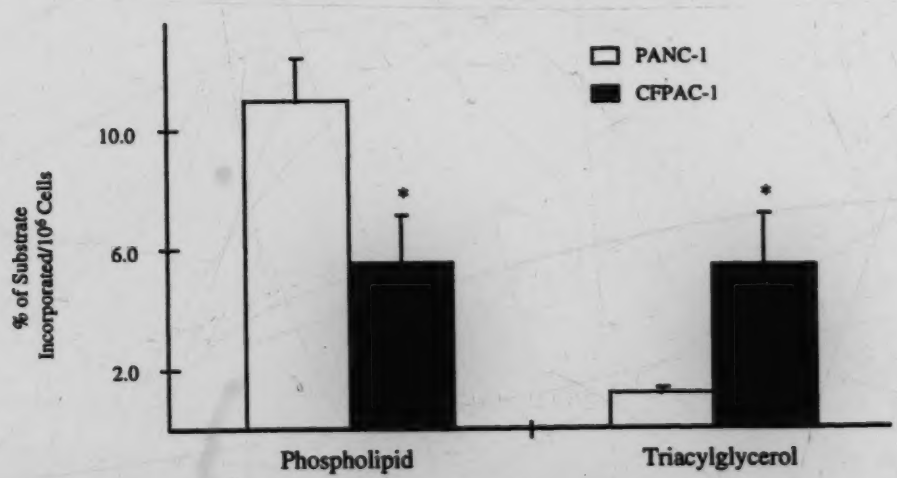

Figure II.2. ^{14}C18:2n-6 incorporation into phospholipid and triacylglycerol of PANC-1 and CFPAC-1 cells. Cells were replated with ^{14}C18:2n-6 for 4 hours. Lipids were extracted and lipid classes separated by TLC. Radioactivity was counted by liquid scintillation spectrometry. Values are the mean ± SEM, n=7. *$p<0.05$.

phospholipids indicated reduced incorporation of ^{14}C18:2n-6 into phosphatidylcholine (3.7% ± 1.20 vs 9.2% ± 0.94, $p<0.05$) and phosphatidylethanolamine (0.6% ± 0.24 vs 1.2% ± 0.16, $p<0.05$) of CFPAC-1 cells in contrast to PANC-1 cells (Figure II.3). Considering the level of substrate incorporation into the individual phospholipids, this data suggests that the reduced incorporation into total phospholipid is largely the result of reduced incorporation into phosphatidylcholine.

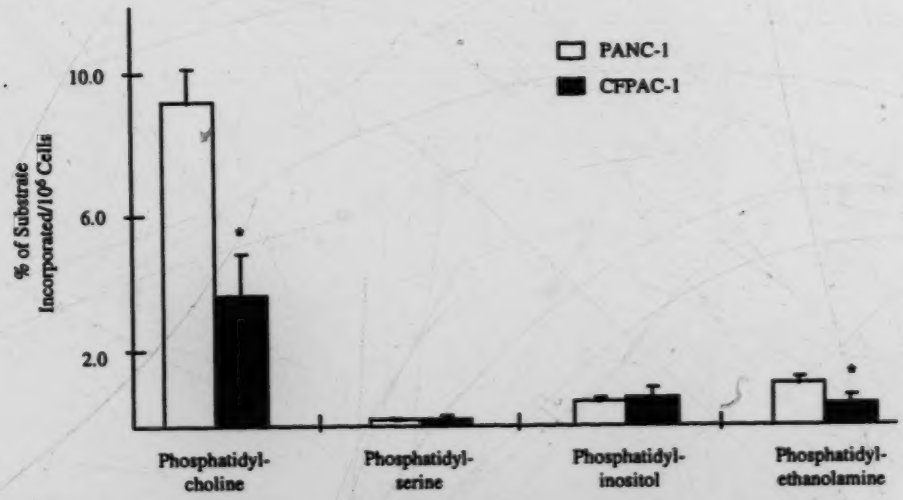

Figure II.3. ^{14}C18:2n-6 incorporation into phospholipids of PANC-1 and CFPAC-1 cells. Cells were replated with ^{14}C18:2n-6 for 4 hours. Lipids were extracted and phospholipids separated by TLC. Radioactivity was counted by liquid scintillation spectrometry. Values are the mean ± SEM, n=6. *$p<0.05$.

3. Time Course of the Incorporation of ^{14}C18:2n-6 into Triacylglycerol and Phospholipids of PANC-1 and CFPAC-1 Cells

At each time point studied, the incorporation of ^{14}C18:2n-6 was higher in CFPAC-1 triacylglycerol ($p<0.001$) in contrast to PANC-1 cells (Figure II.4A).

This suggests that the increased substrate incorporation into this neutral lipid in CFPAC-1 cells is independent of time. The data obtained for PANC-1 cells indicates that there was very little change in the incorporation of ^{14}C18:2n-6 over time into the neutral lipid. Substrate incorporation into total phospholipid did not differ for the cell types until 4 hours when it was reduced ($p<0.05$) in CFPAC-1 cells (Figure II.4B).

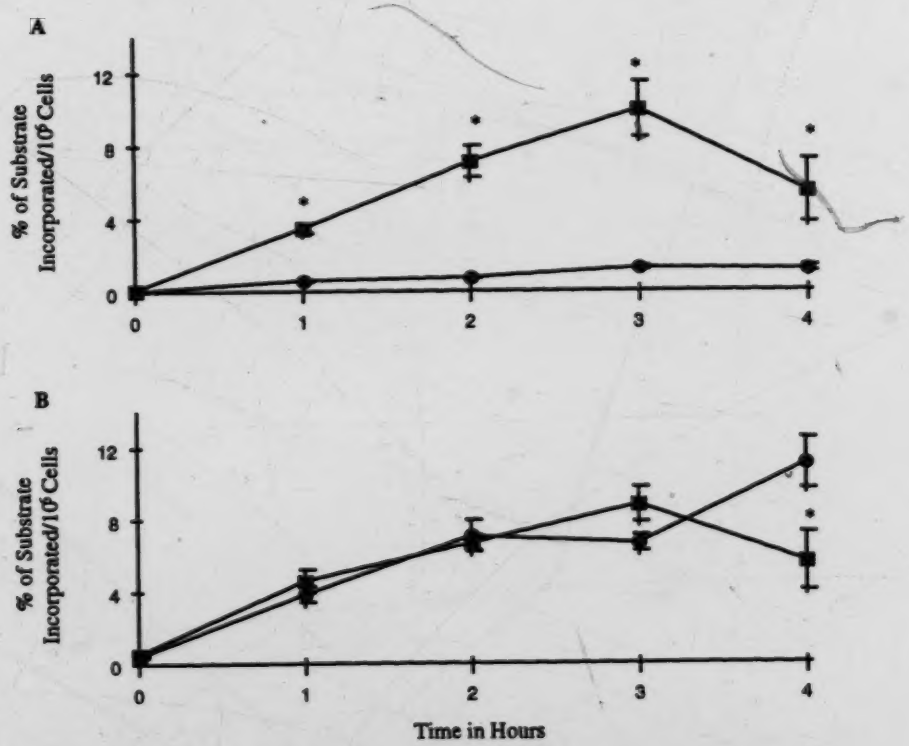

Figure II.4. Time course for incorporation of ^{14}C18:2n-6 over four hours into triacylglycerol (A) and phospholipid (B) of PANC-1 and CFPAC-1 cells. Cells were replated with ^{14}C18:2n-6 for 0, 1, 2, 3 or 4 hours. Lipids were extracted and lipid classes separated by TLC. Radioactivity was counted by liquid scintillation spectrometry. Values are the mean ± SEM, n=5. *$p<0.001$ (A) and $p<0.05$ (B). Circle = PANC-1; square = CFPAC-1.

Figure II.5 represents time course data from Figures II.4A and B analyzed using the SAAM II Biological Modeling Program (Seattle, WA). Data (% of substrate incorporated/10^6 cells) for each time point was analyzed using simple, built-in differential equations to produce rate constants for the movement of the substrate (^{14}C18:2n-6) between lipid pools. Rate constants for the movement of ^{14}C18:2n-6 between phospholipid and triacylglycerol of PANC-1 cells differed ($p<0.01$) whereas in CFPAC-1 cells it did not.

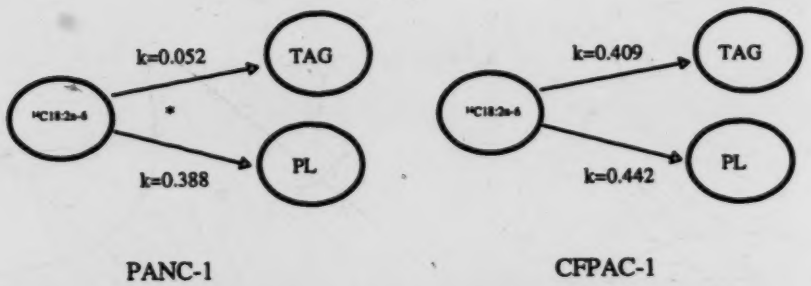

Figure II.5. Kinetic modeling of the rate constants for the incorporation of ^{14}C18:2n-6 over four hours into triacylglycerol and phospholipid of PANC-1 and CFPAC-1 cells. Data from figures 8A and B have been analyzed using the SAAM II Biological Modeling Program (Seattle, WA). Values ($k=hr^{-1}$) are the mean $\pm$ SEM, n=5. *$p<0.01$. TAG = triacylglycerol; PL = phospholipid.

Time courses for substrate incorporation for phosphatidylcholine and phosphatidylethanolamine indicates that the difference between the cell types occurs at 4 hours (Figure II.6). The time course for ^{14}C18:2n-6 incorporation into total phospholipid and phosphatidylcholine follow a similar pattern which implies

that ^{14}C18:2n-6 incorporation into total phospholipid in CFPAC-1 largely reflects alterations occurring in the major membrane phospholipid, phosphatidylcholine.

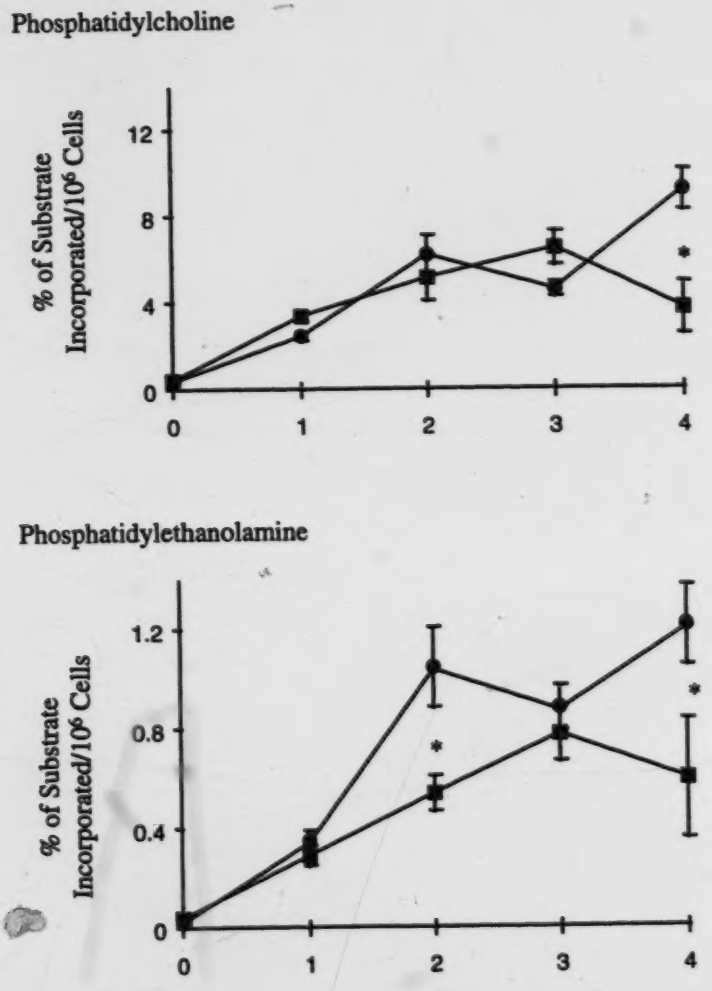

Figure II.6. Time course for incorporation of ^{14}C18:2n-6 over four hours into phospholipids of PANC-1 and CFPAC-1 cells. Cells were replated with ^{14}C18:2n-6 for 0, 1, 2, 3 or 4 hours. Lipids were extracted and phospholipids separated by TLC. Radioactivity was counted by liquid scintillation spectrometry. Values are the mean $\pm$ SEM, n=5. *$p<0.05$.
Circle = PANC-1; square = CFPAC-1.

4. The Effect of CFTR on ^{14}C18:2n-6 Incorporation into Triacylglycerol and Phospholipids

Prior to undertaking ^{14}C18:2n-6 incorporation studies on transfected CFPAC-1 cells, functional studies of chloride conductance were completed. Results indicated that chloride efflux was over 300% higher for cells transfected with the wild-type gene compared to transfection with CFTRΔF508 (1.3 ± 0.13 vs 0.4 ± 0.03, $p<0.001$) (Figure II.7).

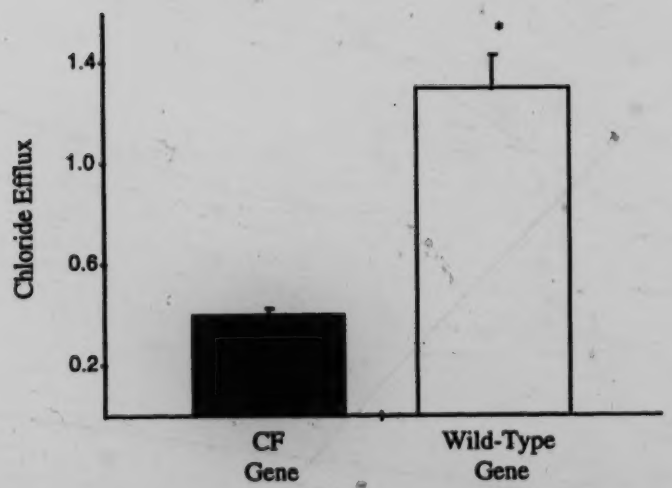

Figure II.7. Chloride conductance of transfected CFPAC-1 cells. Cells transfected with wild-type CFTR or CFTRΔF508 were incubated with $^{36}Cl^-$ for 2 hours. Chloride efflux was measured over 15 second intervals. Values for baseline and stimulated (with forskolin and 3-isobutyl-1-methylxanthine (IBMX)) efflux were obtained. Chloride efflux was calculated as $^{36}Cl^-$ released from the monolayer over time and presented as the Σ released (stimulated)/Σ released (baseline). Values are the mean ± SEM, n=7. *$p<0.001$.

Labeled substrate incorporation into CFPAC-1 cells transfected with the wild-type gene was higher for both total phospholipid (9.1% ± 0.92 vs 6.0% ± 0.60, $p<0.03$) and triacylglycerol (9.2% ± 1.27 vs 4.8% ± 0.45, $p<0.03$) compared to cells transfected with CF gene (Figure II.8). The increased ^{14}C18:2n-6 incorporation into triacylglyerol was an unexpected finding since CFPAC-1 cells exhibited an increased ^{14}C18:2n-6 incorporation into triacylglycerol compared to the wild-type group (Figure II.2). A comparison between transfected CFPAC-1, PANC-1 and CFPAC-1 cells for ^{14}C18:2n-6 incorporation into triacylglycerol

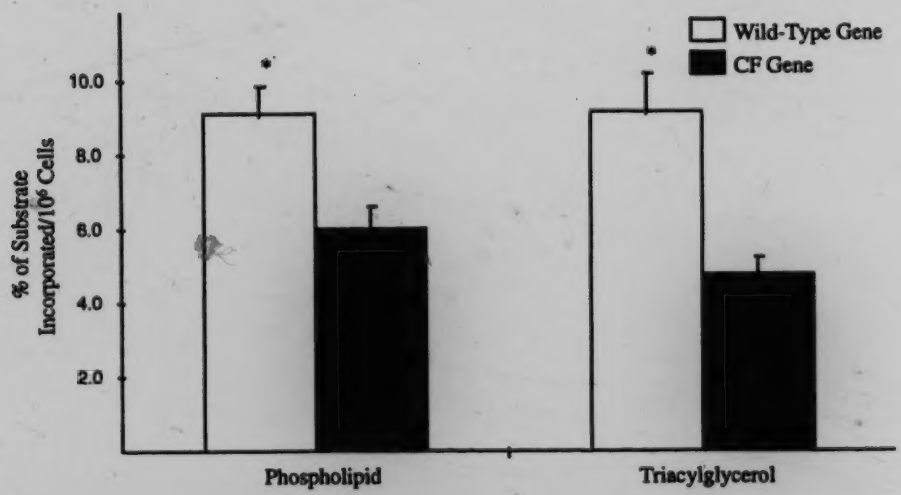

Figure II.8. ^{14}C18:2n-6 incorporation into phospholipid and triacylglycerol of transfected CFPAC-1 cells. Cells transfected with wild-type CFTR or CFTRΔF508 were replated with ^{14}C18:2n-6 for 4 hours. Lipids were extracted and lipid classes separated by TLC. Radioactivity was counted by liquid scintillation spectrometry. Values are the mean ± SEM, n=7 (wild-type gene) and n=4 (CF gene). *$p<0.03$.

(Figure II.9) revealed that all 3 CFPAC-1 cell lines (wild-type gene, CF gene and CFPAC-1) exhibit increased level of substrate incorporation into this neutral lipid compared to PANC-1 cells. Similar illustrations for phospholipid (Figure II.10) and phosphatidylcholine (Figure II.11) are also presented.

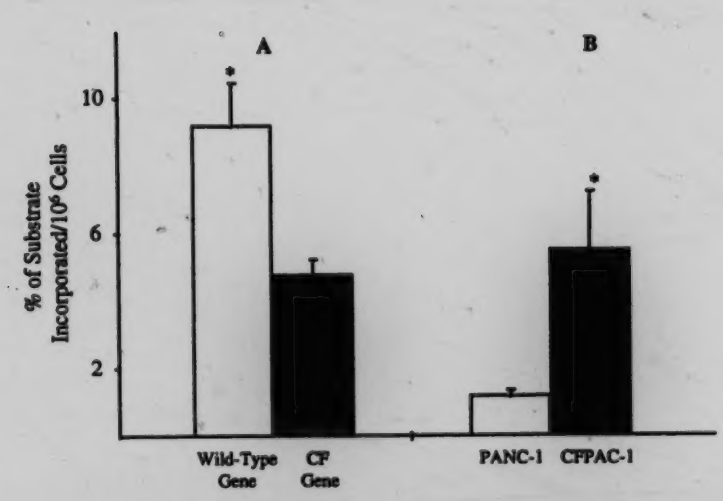

Figure II.9. ^{14}C18:2n-6 incorporation into triacylglycerol: a comparison between transfected (A), PANC-1 and CFPAC-1 (B) cells. Data from figures II.2 and II.8 have been combined to produce this figure. *$p<0.03$, n=7 (wild-type gene) and n=4 (CF gene) (A) and $p<0.05$, n=7 (B).

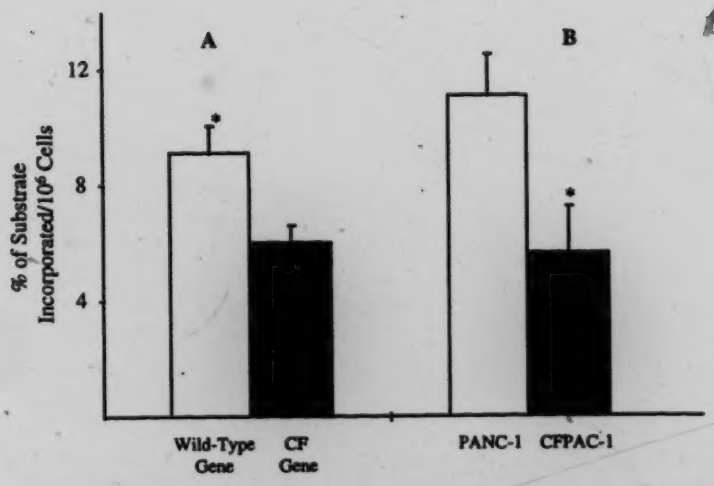

Figure II.10. ^{14}C18:2n-6 incorporation into phospholipid: a comparison between transfected (A), PANC-1 and CFPAC-1 (B) cells. Data from figures II.2 and II.8 have been combined to produce this figure. *p<0.03, n=7 (wild-type gene) and n=4 (CF gene) (A) and p<0.05, n=7 (B).

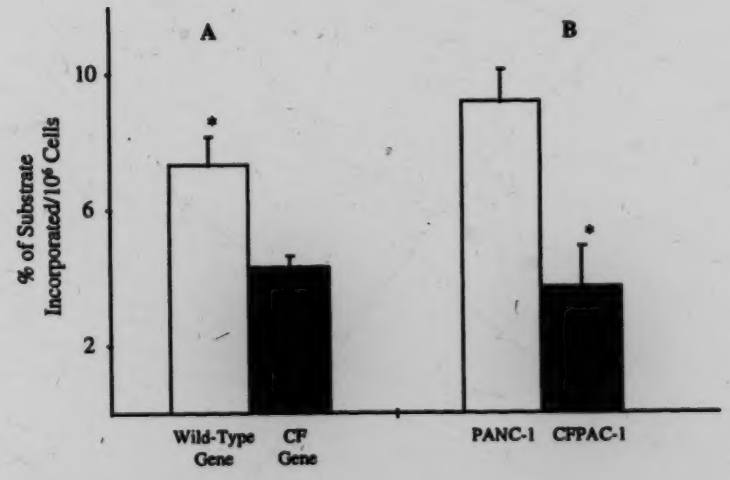

Figure II.11. ^{14}C18:2n-6 incorporation into phosphatidylcholine: a comparison between transfected (A), PANC-1 and CFPAC-1 (B) cells. Data from figures II. 3 and II.11 have been combined to produce this figure. *p<0.03, n=7 (wild-type gene) and n=4 (CF gene) (A) and p<0.05, n=6 (B).

Studies of chloride efflux were performed on PANC-1 and CFPAC-1 cells to compare the extent of the increase in chloride efflux of cells transfected with wild-type gene with respect to PANC-1 cells. No difference was noted in the chloride efflux of PANC-1 and CFPAC-1 cells (Figure II.12). Further, the chloride efflux of PANC-1, CFPAC-1 and CF gene group was similar (0.45 $\pm$ 0.03, 0.50 $\pm$ 0.02 and 0.40 $\pm$ 0.03, respectively) (Figures II.7 and II.12).

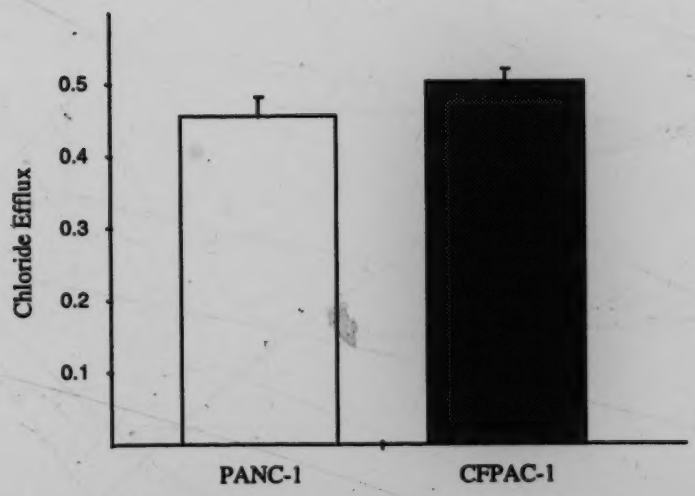

Figure II.12. Chloride conductance of PANC-1 and CFPAC-1 cells. Cells were incubated with $^{36}Cl^-$ for 2 hours. Chloride efflux was measured over 15 second intervals. Values for baseline and stimulated (with forskolin and 3-isobutyl-1-methylxanthine (IBMX)) efflux were obtained. Chloride efflux was calculated as $^{36}Cl^-$ released from the monolayer over time and presented as the Σ released (stimulated)/Σ released (baseline). Values are the mean $\pm$ SEM, n=4. p=NS.

Separation of phospholipids revealed increased ^{14}C18:2n-6 incorporation into phosphatidylchloline (7.3% ± 0.82 vs 4.3 ± 0.33, $p<0.05$), phosphatidylserine (0.2% ± 0.02 vs 0.1% ± 0.01) and phosphatidylinositol (1.2% ± 0.14 vs 0.8% ± 0.06) of the wild-type gene group compared to cells transfected with the CF gene (Figure II.13).

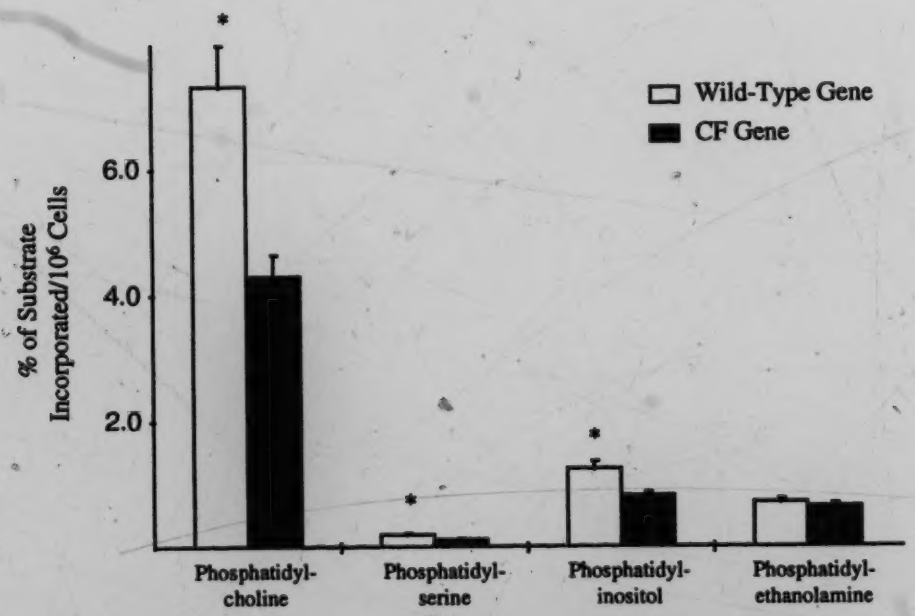

Figure II.13. ^{14}C18:2n-6 incorporation into phospholipids of transfected CFPAC-1 cells. Cells transfected with wild-type CFTR or CFTRΔF508 were replated with ^{14}C18:2n-6 for 4 hours. Lipids were extracted and lipid classes separated by TLC. Radioactivity was counted by liquid scintillation spectrometry. Values are the mean ± SEM, n=7 (wild-type gene) and n=4 (CF gene). *$p<0.03$.

5. Conversion of ^{14}C18:2n-6 to its Desaturation and Elongation Products: Distribution of Labeled Fatty Acid Chains into Total and Individual Phospholipids

Desaturation and elongation of radiolabeled C18:2n-6 was determined in PANC-1, CFPAC-1 and transfected CFPAC-1 cells (Tables II.1 - II.4). The results suggest that all cell types are capable of desaturating-elongating ^{14}C18:2n-6 indicating the presence active Δ6- and Δ5-desaturase enzyme systems.

In comparison to PANC-1 cells, CFPAC-1 cells exhibited increased level of ^{14}C18:2n-6 in phospholipid, phosphatidylcholine and phosphatidyl-ethanolamine (Tables II.1 and II.2). The increase in labeled C18:2n-6 in phospholipids disagrees with previous results and may be related to the shaking of cell samples. The cell samples for this study were gently shaken to allow exposure of the basolateral membrane to the medium to assess whether attachment of this side of the membrane to an artificial surface (culture dish) produces a difference in the incorporation of ^{14}C18:2n-6. Since the results for CFPAC-1 and CF gene groups are similar for all three fractions examined (dpm/10^6 cells) and have been for other studies performed in this research project, it is believed that the discrepancy in the ^{14}C18:2n-6 level observed in phospholipids of CFPAC-1 cells reflects reduced incorporation into the PANC-1 group, rather than a genuine increase in CFPAC-1 group.

There were no differences between the distribution of the desaturation-elongation products of ^{14}C18:2n-6 in all three fractions studied in the PANC-1 and CFPAC-1 groups, with the exception of the trienes which were decreased in

the total phospholipid and increased in the phosphatidylethanolamine fractions of CFPAC-1 cells.

In the phospholipid fraction, the expression of the wild-type gene resulted in an increased level of ^{14}C18:2n-6 and reduced level of ^{14}C20:4n-6 in comparison to the CF gene group (Table II.3). Separation of phospholipids revealed increased ^{14}C18:2n-6 in both phosphatidylcholine and phosphatidylethanolamine of the wild-type gene group with no difference in the level of ^{14}C20:4n-6 (Table II.4). The longer chain desaturated product ^{14}C22:5n-6 was increased in phosphatidyl-ethanolamine of the wild-type gene group.

Table II.1. The Conversion of ^{14}C18:2n-6 to its Desaturation-Elongation Products in Phospholipid of PANC-1 and CFPAC-1Cells

Fatty Acid	Distribution of Radioactivity (%) PANC-1	CFPAC-1
18:2n-6	57±1.7	69±4.6*
18:3n-6 & 20:3n-6	15±0.2	10±0.8*
20:4n-6	18±1.0	15±0.9
22:5n-6	10±1.6	6±3.4

Cells were replated with ^{14}C18:2n-6 for 4 hours. Lipid was extracted and phospholipid separated by TLC. Fatty acids were methylated followed by double bond separation using argentation TLC. Radioactivity was counted by liquid scintillation spectrometry. Values represent the mean ±SEM, n=4 (PANC-1) and 3 (CFPAC-1). Comparisons between cell types are significantly different at $p<0.05$ (*).

Table II.2. The Distribution of ^{14}C18:2n-6 Desaturation-Elongation Products[1] in Phospholipids of PANC-1 and CFPAC-1 Cells

Fatty Acid	Phosphatidyl-choline		Phosphatidyl-ethanolamine	
	PANC-1	CFPAC-1	PANC-1	CFPAC-1
18:2n-6	469±48	825±133*	76±7	155±19*
18:3n-6 & 20:3n-6	119±11	110±23	34±2	53±6*
20:4n-6	114±14	99±25	103±18	151±22
22:5n-6	32±2	30±8	18±2	16±1

Cells were replated with ^{14}C18:2n-6 for 4 hours. Lipid was extracted and phospholipids separated by TLC. Fatty acids were methylated followed by double bond separation using argentation TLC. Radioactivity was counted by liquid scintillation spectrometry. Values (dpm/10^6 cells) represent the mean ± SEM, n=4 (PANC-1) and 3 (CFPAC-1). Comparisons between cell types are significantly different at $p<0.05$ (*).
[1]Values x 10^{-2}.

Table II.3. The Conversion of ^{14}C18:2n-6 to its Desaturation-Elongation Products in Phospholipid of Transfected CFPAC-1 Cells

Fatty Acid	Distribution of Radioactivity (%)	
	Wild-Type Gene	CF Gene
18:2n-6	71±2.2*	55±3.2
18:3n-6 & 20:3n-6	12±0.7	11±0.9
20:4n-6	10±0.8*	28±3.6
22:5n-6	7±1.0	6±1.3

Cells were replated with ^{14}C18:2n-6 for 4 hours. Lipid was extracted and phospholipid separated by TLC. Fatty acids were methylated followed by double bond separation using argentation TLC. Radioactivity was counted by liquid scintillation spectrometry. Values represent the mean ±SEM, n=4. Comparisons between cell types are significantly different at $p<0.05$ (*).

Table II.4. The Distribution of ^{14}C18:2n-6 Desaturation-Elongation Products[1] in Phospholipids of Transfected CFPAC-1 Cells

Fatty Acid	Phosphatidyl-choline		Phosphatidyl-ethanolamine	
	Wild-Type Gene	CF Gene	Wild-Type Gene	CF Gene
18:2n-6	1734±261*	756±232	281±42*	82±22
18:3n-6 & 20:3n-6	170±16	156±44	68±9	43±14
20:4n-6	129±16	145±54	107±18	107±47
22:5n-6	74±23	41±10	39±6*	17±5

Cells were replated with ^{14}C18:2n-6 for 4 hours. Lipid was extracted and phospholipids separated by TLC. Fatty acids were methylated followed by double bond separation using argentation TLC. Radioactivity was counted by liquid scintillation spectrometry. Values (dpm/10^6 cells) represent the mean ± SEM, n=4. Comparisons between cell types are significantly different at $p<0.05$ (*).
[1]Values x 10^{-2}.

D. Discussion

The objective of the present research was to investigate whether the ΔF508 mutation in the CFTR reduces the incorporation of the essential fatty acid C18:2n-6 into phospholipids of CF cells. The results indicate that the defective CFTR reduced incorporation of C18:2n-6 into phospholipids of CFPAC-1 cells. This finding provides an important insight into an aspect of the CF defect and its relationship to the characteristic essential fatty acid profile. The results also suggest that CF may not represent a 'true' essential fatty acid deficiency but rather results from impaired intracellular utilization of C18:2n-6.

1. Transfected CFPAC-1 Cells in the Study of CFTR Function

The CFPAC-1 pancreatic adenocarcinoma cell line expressing the ΔF508 mutation (Schoumacher et al., 1990) stably transfected using the retrovirus-mediated gene transfer method (Drumm et al., 1990) has been used previously to assess the cellular function of the CFTR (Drumm et al., 1990; Bradbury et al., 1992; Kersting et al., 1994; Dunn et al., 1994). Comparing transfected cells with the genetically matched parental cells ascertain the function of the CFTR by eliminating both between-cell and between-individual variability. As a result, the differences measured between the two cell lines can be attributed to the CFTR alone with more confidence. These cell lines were utilized in the present study to investigate the role of the CFTR in producing the characteristically low C18:2n-6 levels in the phospholipids of CF cells. The results demonstrate a relationship between the CFTR and the metabolism of C18:2n-6 in CF.

2. Chloride Conductance of PANC-1 and CFPAC-1 Cells

The PANC-1 cells (Leiber et al., 1975) were utilized in this study to serve as control against the corresponding CFPAC-1 (CF) cell line. There was no difference in the chloride conductance of normal and CF cells (Figure II.10). Studies suggest that the pancreatic adenocarcinoma cell line (PANC-1) rarely expresses the CFTR gene, despite high levels of the CFTR protein found in the primary pancreatic duct cells from which they are derived (Smith et al., 1995). Furthermore, recent efforts at transfecting this cell line to stably synthesize high levels of CFTR mRNA resulted in little expression of the CFTR protein (Smith et

al., 1995). These findings may explain the lack of difference in chloride conductance found between CFPAC-1 and the correspondent PANC-1 cell lines. It has also been demonstrated that in CF the pattern of CFTR expression in tissues does not necessarily conform to the disease pathology (Tizzano et al., 1993 and 1994; Smith et al., 1995). Thus, although there was no difference noted in the chloride efflux, the use of PANC-1 cells should allow valid comparisons to be made with respect to differences in the normal versus diseased state.

4. Incorporation of Linoleic Acid into Phospholipid and Triacylglycerol

In vivo, the majority of fatty acids transported to tissues comprises free fatty acid or triacylglycerols of very low density lipoproteins and chylomicrons (Spector, 1992). The triacylglycerols contained in the lipoproteins are hydrolyzed by lipoprotein lipase, releasing the fatty acids for uptake into tissues. Thus, for the most part, it is the fatty acid (subsequently bound to plasma albumin) that is presented to the cells. To mimic this physiological condition, in vitro studies of fatty acid utilization are performed by incubating cells with free fatty acids bound to albumin (reviewed by Rosenthal, 1987 and Spector et al., 1981 and Spector, 1992; Kang et al., 1992). In the present study, free C18:2n-6 bound to albumin was also used to imitate the in vivo circumstance.

The results of the present study are similar to those of previous studies in which have shown that blocking chloride channels in normal human airway epithelial cells decreased incorporation of fatty acids including C18:2n-6 into phospholipids while increasing incorporation of C18:2n-6 into triacylglycerol

(Kang et al., 1992). This suggests that the effect of the CFTR on C18:2n-6 incorporation into phospholipids observed in the present study may be related to chloride conductance. The results show increased C18:2n-6 incorporation into phospholipids of both the PANC-1 and the wild-type gene groups. However, the difference in the chloride conductance of these groups suggests that the incorporation of C18:2n-6, although related to the CFTR, appears to be independent of its role as a chloride channel. However, it is also possible that the chloride channel blocker functions differently than the CFTR and its effect on fatty acid incorporation was related to factors such as chloride concentration (Kang et al., 1992) rather than chloride conductance. The possibility also exists that the CFTR regulates other cellular functions, thereby exerting its effect on C18:2n-6 incorporation. Thus, although the importance of functional CFTR is evident for the normal intracellular utilization of C18:2n-6, whether this is related to the chloride channel function or due to alterations in other functions of the CFTR or in the functions mediated by the CFTR, as yet undefined, is not known.

The high level of C18:2n-6 incorporation into the neutral lipid triacylglycerol in the transfected and non-transfected CFPAC-1 cell lines (Figure II.9) implies that this effect is not related to the CFTR but occurs for other reasons. Whether this increase is related to an enhanced triacylglycerol synthesis to accommodate an influx of fatty acid or this neutral lipid pool acts as a trap for the released acyl chains from phospholipids (Chakravarthy et al., 1986) is not known. Blocking chloride channels also resulted in increased incorporation of C18:2n-6 into triacylglycerol (Kang et al., 1992). Thus, it is possible that

impairments in chloride conductance, as the primary defect in CF, may lead to irreversible changes in cellular metabolism which do not respond to the correction of the genetic defect. While the molecular basis of CF has been studied extensively, the biochemical connections between the defective CFTR and the multifaceted functional anomalies observed in CF cells remains unclear (Liedke, 1992; Tizzano et al., 1993 and 1994; Smith et al., 1995). Hence, all aspects of the CF phenotype may not be complemented by the expression of wild-type CFTR (Drumm et al., 1990).

4. Total Linoleic Acid Incorporation into Cellular Lipids

Total substrate incorporation into cellular lipids (phospholipids + neutral lipid) at four hours was similar for the PANC-1, CFPAC-1 and CF gene groups. However, expression of the wild-type CF gene resulted in increased total C18:2n-6 incorporation into cellular lipids (64% higher than the CF gene group). These results suggest that the mechanism(s) involved may be both an effect on the transport of the fatty acid across the plasma membrane and related to the intracellular handling of the essential fatty acid. Kinetic modeling of the time course data was used to illustrate the partitioning of linoleic acid between relevant cellular lipid pools in PANC-1 and CFPAC-1 cells (Figure II.5). Results indicate that CF cells have lost control over the intracellular distribution of linoleic acid between phospholipid and triacylglycerol. The fact that transfection with the wild-type CFTR had a positive effect on C18:2n-6 incorporation into phospholipid, but not triacylglycerol, suggests that this abnormal distribution of

the essential fatty acid between lipid pools is regulated in part by the CFTR. Furthermore, considering the similarity in the chloride efflux of PANC-1, CFPAC-1 and CF gene groups and the large increase in the chloride conductance upon expression of the wild-type gene suggests a role for chloride conductance in altering the transport of C18:2n-6. The potential role of chloride conductance in the transport of fatty acids across plasma membrane has also been suggested by previous work (Kang et al., 1992).

5. Desaturation-Elongation of Linoleic Acid

Δ6- and Δ5-desaturase enzymes are involved in the metabolism of C18:2n-6 and are necessary for the synthesis arachidonic acid (C20:4n-6) which is an essential requirement for the synthesis of phospholipids and eicosanoids (Jeffcoat and James, 1984; Sardesai, 1993; Simplicini and Valle, 1994). If the desaturation pathway functions normally, it would be predicted that a deficiency of C18:2n-6 would cause a decrease in the availability of C20:4n-6. The results of the conversion of radiolabeled C18:2n-6 to its desaturation-elongation products in total phospholipid indicate no differences in C20:4n-6 level between PANC-1 and CFPAC-1 cells. However, in comparison to the CF gene group, a reduction in the level of C20:4n-6 is observed in the wild-type group. The decrease in C20:4n-6 may be related to the increase in the esterification of its precursor, C18:2n-6 into phospholipids. This suggests that in CF there may be an enhanced desaturation-elongation of C18:2n-6 as a result of increased availability or rather than increased desaturation-elongation, just the availability of C18:2n-6 for its further

metabolism to C20:4n-6. Hence, normalization of the genetic defect causes an increase in the incorporation of C18:2n-6 into phospholipid (as seen in this study) which leads to a reduced availability of C18:2n-6 for desaturation-elongation reactions, thereby reducing the overall level of C20:4n-6 into phospholipid. Whether this reduced level is comparable to control cells (see Results) needs to be addressed.

There were no differences in the level of C20:4n-6 in phosphatidylcholine and phosphatidylethanolamine in all cell types studied. This suggests that the reduction in C20:4n-6 seen in total phospholipid of the wild-type gene group is not reflected in either of these phospholipids and may indicate differences in other phospholipids not examined. However, the ratio of trienes to tetraenes was similar for the CFPAC-1 and CF gene groups for both phosphatidylcholine (1.10 and 1.08, respectively) and phosphatidylethanolamine (0.35 and 0.41, respectively) and was higher for the wild-type gene group (1.32 for phosphatidylcholine and 0.64 for phosphatidylethanolamine) which supports the overall reduction in C20:4n-6 observed in the total phospholipid fraction of the wild-type gene group and suggests a critical link between the defective CFTR and the altered essential fatty acid profile of CF.

Based on the fatty acid profile of tissue and plasma lipids, several studies have reported normal to increased levels of C20:4n-6 despite low C18:2n-6 levels in CF (Chase and Dupont, 1978; Lloyd-Still et al., 1981; Hubbard and Dunn, 1980; Rogiers et a., 1984; Farrell et al., 1985; Parsons et al., 1988; Christophe et al., 1992; Lawrence and Sorrell, 1993), although low levels of C20:4n-6 have also

been observed (Clandinin et al., 1995). It has been suggested that arachidonic acid plays a role in the pathology of CF (Strandvik, 1988 and 1992). C20:4n-6 and/or its metabolites, prostaglandins, thromboxanes and leukotrienes, have been shown to regulate chloride transport (Eling et al., 1986; Leikauf et al., 1986; Anderson and Welsh, 1990; Calderaro et al., 1991), increase production of mucus in both gastrointestinal and respiratory tracts (Marom et al., 1981; Lamorte et al., 1986) and involved in the regulation of the β-adrenergic response (Davis, 1986). In CF, the release of C20:4n-6 occurs at a steady rate, irrespective of the factors normally known to inhibit its release such as dexamethasone (Carlstedt-Duke et al., 1986). Further, the synthesis of eicosanoids in CF has been found to be greater than in control subjects (Chase and Dupont, 1978; Stead et al., 1986). It has been postulated that this increase in C20:4n-6 release results from a defective regulation of phospholipase A_2, an enzyme which is also rate limiting in the production eicosanoids (Strandvik, 1988 and 1992). These observations and those of the present study suggest CF may not represent a 'true' essential fatty acid deficiency but rather exemplifies a defective regulation of essential fatty acid metabolism. Hence, the finding of normal levels of C20:4n-6 despite low C18:2n-6 levels of membrane lipids in CF may be explained by the results of this study which have shown: 1) reduced incorporation of C18:2n-6 into phospholipids and 2) the intracellular availability of C18:2n-6 for desaturation-elongation reactions.

6. Summary

The regulation of the fatty acids found in membrane lipids is due to the coordinated efforts of the enzymes involved in their esterification into specific cellular lipid pools (Rosenthal, 1987; Sprecher, 1992). The results of this study have indicated that CFPAC-1 cells exhibiting the ΔF508 mutation in the CFTR have lost this ability to appropriately partition the essential fatty acid C18:2n-6 between phospholipid and triacylglycerol pools. Subsequently, the available C18:2n-6 is desaturated and elongated to ensure normal to increased levels of C20:4n-6 commonly found in CF. Whether the availability of C18:2n-6 or an enhanced metabolism of this fatty acid occurs to ensure these levels of C20:4n-6 is not clear. These observations suggest an important insight into the altered essential fatty acid profile consistently reported in CF which may be explained by the defective CFTR.

E. References

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Chapter III

General Summary and Discussion

A. Summary

The first hypothesis was that the C18:2n-6 content of CF phospholipids will be lower in comparison to control cells. The CFPAC-1 cells homozygous for the ΔF508 mutation in the CFTR and the corresponding control (PANC-1) cells were utilized to test this hypothesis. The results of this study supported this hypothesis by the observation of low C18:2n-6 level in phospholipids, specifically in phosphatidylcholine and phosphatidylethanolamine of CFPAC-1 cells in comparison to PANC-1 cells grown in standard culture conditions. Furthermore, normal to increased levels of C20:4n-6 were found in the phospholipids examined. These findings correspond with the characteristic essential fatty acid profile of CF.

The second hypothesis was that the CFTRΔF508 reduces the incorporation of C18:2n-6 into phospholipids of CF cells. Utilizing the PANC-1 and CFPAC-1 cell lines it was found that radiolabeled C18:2n-6 incorporation was decreased into phospholipids and increased into triacylglycerol of CFPAC-1 cells. With respect to specific phospholipids, we found reduced incorporation of C18:2n-6 into phosphatidylcholine and phosphatidylethanolamine of CFPAC-1 cells in comparison to PANC-1. The time course data for phospholipid and triacylglycerol revealed a loss of control of the intracellular distribution of C18:2n-6 in CFPAC-1 cells. These findings suggested that rather than a deficiency, the essential fatty acid status of CF cells represent a defective utilization of C18:2n-6.

The third hypothesis was that the transfection with wild-type CFTR will increase the incorporation of C18:2n-6 into phospholipids of CF cells. CFPAC-1 cells transfected with either CF (ΔF508) gene or wild-type CF gene were employed to investigate the function of the CFTR in C18:2n-6 incorporation into cellular lipids. The results confirmed this hypothesis by the finding that correction of the genetic defect resulted in increased incorporation of C18:2n-6 into phospholipids, specifically into phosphatidylcholine, phosphatidylserine and phosphatidylinositol. The finding of increased C18:2n-6 incorporation into triacylglycerol of the wild-type gene group was not only unexpected, but was found to be similar in all CFPAC-1 cells (transfected and non-transfected). These observations indicated that the intracellular partitioning of C18:2n-6 into cellular lipid pools is regulated in part by the CFTR.

The conversion of radiolabeled C18:2n-6 to its desaturation-elongation products was also examined in PANC-1, CFPAC-1 and transfected CFPAC-1 cells to examine whether a defect in these enzyme systems contributes to the defective C18:2n-6 metabolism in CF. There was increased C18:2n-6 and decreased C20:4n-6 levels in the phospholipids of the wild-type gene group which suggested that the low C18:2n-6 and normal to high C20:4n-6 levels in CF phospholipids may be due to the availability of C18:2n-6 as a result of its decreased esterification into phospholipids. Thus, C18:2n-6 is not deficient but accessible for conversion to its desaturation-elongation products to ensure appropriate levels of C20:4n-6 in CF phospholipids.

B. Discussion

CF is a complex genetic disease exhibiting a variety of pathological alterations at the clinical and cellular levels. The gene causing CF has been isolated with the gene product, the CF transmembrane conductance regulator (CFTR) identified as well as characterized. It is generally accepted that the CFTR is a cAMP-dependent chloride channel in the apical membrane of epithelial cells. However, it appears that the function of the CFTR is not limited to chloride conductance and it may have other regulatory roles in cellular metabolism.

Abnormal essential fatty acid profile of plasma and tissue lipids is characteristic of CF and has been reported for decades. Although many speculations have been made with respect to the basis for this abnormality, its cause remains unclear. For the first time, the results of the present study suggest a relationship between the CFTR and the essential fatty acid abnormality in CF. The results indicate that the typically low C18:2n-6 and normal C20:4n-6 levels of CF phospholipids may be explained by the CFTR which appears to regulate both the transport of C18:2n-6 across the plasma membrane and the intracellular partitioning of C18:2n-6 between phospholipid and triacylglycerol pools. These findings provide an important insight into the function of the CFTR and the essential fatty acid status of patients with CF, acknowledging the pleiotropic nature of the disease. Based on these observations, the essential fatty acid profile of CF, rather than a deficiency, represents an altered utilization of C18:2n-6. Hence, CF results in alterations in essential fatty acid metabolism which transcend the nutritional consequences of the disease.

The mechanism(s) by which the CFTR impairs the incorporation of C18:2n-6 into phospholipids remains to be investigated. Future studies to address the metabolism of C18:2n-6 in healthy (non-CF) cells which ordinarily express large amounts of CFTR are also indicated.

APPENDIX I

Essential Fatty Acid Composition of PANC-1 and CFPAC-1 Cells

Table 1. Average C18:2n-6 and C20:4n-6 Composition of Phospholipids of PANC-1 and CFPAC-1 Cells

Cell Type	Fatty Acid	PC	PE	PI	PS
		% w/w			
PANC-1	18:2n-6	0.6±0.03	0.7±0.08	0.8±0.24	0.6±0.14
	20:4n-6	1.6±0.03	5.9±0.09	5.2±0.47	1.1±0.06
CFPAC-1	18:2n-6	0.4±0.01*	0.3±0.01*	0.3±0.06	1.0±0.11*
	20:4n-6	1.6±0.02	9.0±0.17**	10.6±0.10**	2.1±0.10**

Lipid was extracted from confluent monolayers of cells and phospholipids separated by TLC. Fatty acids were methylated then subjected to gas chromatography. Values represent the mean ± SEM, n=4. Comparisons between cell types are significantly different at $p<0.05$ (*) and $p<0.002$ (**). PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine.